The aetiopathogenesis, cardiovascular and metabolic complications, and pharmacogenomics of Addison's disease in South Africa

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appreciation, in my view, stems from a previous lack of interest in this country for what might have previously been considered an orphan disease.

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Dr Ian Louis Ross

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Abbreviations

<	less than
>	greater than
®	registered trademark
3BHSD	3 beta-hydroxysteroid-dehydrogenase
A.T.P.	adenosine triphosphate
AAAS	Achalasia-Addisonianism-Alacrima-Syndrome
Ab's	antibodies
ACA	adrenocortical autoantibodies
ACTH	adrenocorticotrophic hormone
Ad4BP	Adrenal 4-binding protein
AdP	Adrenogonadal primordium
AHC	Adrenal hyperplasia congenital
AIDS	Acquired Immune Deficiency Syndrome
AIRE	autoimmune regulator
ALD	adrenoleukodystrophy
ANOVA	analysis of variance
anti-Tg	thyroglobulin autoantibodies
anti-TTg	tissue transglutaminase autoantibodies
AOR	adjusted odds ratio
APC	antigen presenting cell
APCs	antigen presenting cells
apo	apoprotein
APS	autoimmune polyglandular syndrome
AR	Androgen receptor
ATP	adult treatment panel
AUC	area under the curve
Bcl-xL	bcl-2 proto-oncogene
BMD	bone mineral density
BMI	body mass index
bp	base pairs
BRISK	Coronary Heart Disease Risk Factor Study in the African Population
C_{min}	trough concentration
c.v.	coefficient of variation
CAH	congenital adrenal hyperplasia
CAR	cortisol awakening response
CBG	cortisol binding globulin
CETP	cholesteryl ester transfer protein
Chi^2	Chi-squared test
CI	confidence interval
CIMT	carotid intimal-medial thickness
Cited2	transcriptional co-activator CREB-binding protein/P 300-interacting transactivator with ED-rich tail
CM	chylomicrons
C_{max}	peak concentration
CMV	cytomegalovirus

CORIS	Coronary Risk Factor Study
CRISIC	Coronary Heart Disease Risk Factors in the Coloured Population of the Cape Peninsula
CRP	C-reactive protein
CT	computed tomography
CTL	cytotoxic T-lymphocyte cells
CTLA-4	Cytotoxic T lymphocyte antigen-4
CV	cardiovascular
CVD	cardiovascular disease
CYP 17	17 α-hydroxylase
dATP	deoxyadenosine triphosphate
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1
DC	dendritic cell
dCTP	2'-deoxyctidine 5'-triphosphate
DECODE	Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe
DGGE	denaturing gradient gel electrophoresis
dGTP	deoxyguanosine triphosphate
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDIC	Epidemiology of Diabetes Intervention and Complications Study
EDTA	ethylene diaminetetraacetic acid
EET	epoxyeicosatrienoic acids
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Emx2	empty spiracles 2
EURODIS	European Organisation for Rare Diseases
FA	fatty acids
FADD	fas-associated death domain
FadE	Foetal zone specific Sf1 enhancer
FFA	free fatty acids
FIELD	Fenofibrate Intervention ad Event Lowering in Diabetes
FOXP3	Forkheadbox P3
GAD 65	glutamic acid decarboxylase 65
GC	glucocorticoid
GCR	glucocorticoid receptor
GCRs	Glucocorticoid receptors
GCs	glucocorticoids
GH	growth hormone
Gli	glioma associated oncogen homolog
HBA_1C	glycosylated haemoglobin
HCG	human chorionic gonadotropin
HDL	high density lipoprotein

HDLC	high density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus-1
HLA	Human lymphocyte antigen
HOMA-IR	homeostasis model assessment-insulin resistance
Hoxa1	homeobox A 1
HPA	hypothalamic-pituitary adrenal
hs-CRP	highly sensitive C reactive protein
HWE	Hardy Weinberg Equilibrium
ICA 69	islet-cell autoantibodies
IDL	Intermediate dense lipoprotein
IGF II	Insulin like growth factor II
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-2	Interleukin-2
IPEX	Immune Dysregulation Polyendocrinopathy Enteropathy X-linked Syndrome
IQR	interquartile range
ITAM	immuno receptor tyrosine-based activation motif
IU/L	International units per litre
JDF	Juvenile Diabetes Foundation
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoprotein
LDLC	low density lipoprotein cholesterol
LH	luteinising hormone
Lh9	lim homeobox gene 9
Lim1	Lin 11 Islet 1 and Mec-3 homeobox gene 1
lkβ	specific inhibitor of transcription factor nuclear factor kβ
MEC	medullary epithelial cell
MHC	Major histocompatibility complex
MIC-A	Major histocompatibility class-related
min	minutes
MMTV	Mouse mammary tumour virus
MRI	magnetic resonance imaging
mRNA	messager RNA
N	number
N	number
NALP1	Nacht leucine-rich repeat protein 1
NCEP	National Cholesterol Education Programme
NEFA	non-esterified fatty acids
NF-κβ	Nuclear factor Kappa Beta
NGF	nerve growth factor
NGFI-B	transient nerve growth factor IB-like
NHLS	National Health Laboratory Service
NHANES	National Health and Nutrition Examination Survey
NK cells	Natural killer cells
NR0B1	Critical region on the X chromosome, gene-1
NTX	N-telopeptides

OR	odds ratio
P450 scc	P 450 Side Chain Cleavage Enzyme
P-450 scc	P-450 side chain cleavage enzyme
P450c17	17 α-hydroxylase
P450c21	21 hydroxylase
Pbx1	pre--B-cell Leukaemia Homeobox 1
PCR	polymerase chain reaction
POF	premature ovarian failure
PROCAM	Prospective Cardiovascular Munster Study
PTCH1	Patched 1
PTPN22	protein tyrosine phosphatase non-receptor type 22
r	correlation coefficient
RBG	random blood glucose
RFLP	Restriction fragment length polymorphism
SAA	serum amyloid a
SD	standard deviation
SEM	standard error of the mean
Serum K	Serum potassium
Serum Na	Serum sodium
Sf1	Steroidogenic factor 1
SHBG	sex hormone binding globulin
shh	Sonic hedgehog homolog
SIADH	Syndrome of inappropriate antidiuretic hormone
SLE	systemic lupus erythematosus
SMO	Smoothened
SNP	single nucleotide polymorphism
SNPs	single nucleotide polymorphisms
StAR	Steroidogenic acute regulatory protein
StCA	Steroid cell autoantibodies
T	testosterone
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
T4	Thyroxine
TC	total cholesterol
TCR	T-cell receptor
TG	triglycerides
TGFβ	transforming growth factor β
TM	trademark
t_{max}	time to peak
t_{min}	time to trough
TNFα	Tumour necrosis factor α
TNFβ	Tumour necrosis factor β
TSH	thyroid stimulating hormone
TSST	Trier Social Stress Test
UFC	Urinary free cortisol
UKPDS	United Kingdom Prospective Diabetic Study
USA	United States of America

V	variable region
VEGF	vascular endothelial growth factor
VLCFA	very long chain fatty acids
VLDL	very low density lipoprotein
Wnt 4	Wingless-type MMTV integration site family member 4
WOSCOPS	West of Scotland coronary prevention study
Wt-1	Wilm's tumour 1
xg	centrifugal accelerations related to gravity

Abstract

Introduction

This thesis aimed to address a number of unanswered research questions in Addison's Disease: investigate whether autoimmunity is the predominant cause of Addison's disease in South Africa and if a human leukocyte (HLA) DQ antigen association exists; the extent to which lipids, lipoproteins and biochemical markers of cardiovascular disease are abnormal; the degree to which replacement doses of hydrocortisone are supra-physiological; the impact of glucocorticoid receptor (GCR) polymorphisms on risk factors, markers of cardiovascular disease and replacement doses of hydrocortisone.

Patients

A national database of patients with Addison's disease was compiled from primary care, referral centres and private practices. 148 patients {97 of European descent (white), 34 of mixed ancestry, 5 Asian and 12 black African} were matched with controls for gender and ethnicity.

Methods

Demographic and clinical data were elicited using questionnaires. Anthropometric data were recorded and blood was drawn. The causes of Addison's disease were investigated using a specific algorithm. Lipids, lipoproteins and markers of cardiovascular disease were assessed. Salivary cortisol day curves were evaluated in 31 Addison's patients on usual hydrocortisone doses and control subjects. The role of the GCR polymorphisms was explored to determine its influence on metabolic parameters and hydrocortisone dose.

Results

Fifty one percent of patients' Addison's disease was autoimmune in origin. Either 21-hydroxylase or adrenocortical autoantibodies were present in 50% of the

cohort, while 23% had both. None of the Asian or black patients had detectable evidence of autoimmune disease. Overall 8% had tuberculosis, 4% had adrenoleukodystrophy, 1% had ACTH-resistance syndrome and 6% had X-linked adrenal hypoplasia. HLA DQB1*0201 predominated in the autoimmune group. Almost 50% had hypertriglyceridaemia, 65% had hypercholesterolaemia, about 75% had low high density lipoprotein cholesterol (HDLC) and 75% had elevated low density lipoprotein cholesterol (LDLC). Highly sensitive C-reactive protein (hs-CRP) was increased in both white and mixed ancestry patients, compared to controls. The Framingham risk >20% in 10 years was found in 36% of the cohort. The Addison's patients had significantly higher first and second peak salivary cortisol concentrations, and median and interquartile range (IQR) salivary cortisol area under the curve (AUC) than the controls' endogenous cortisol profiles. The AUC correlated with the peak salivary cortisol concentrations in patients, (r = 0.87; p = 0.0001) and controls (r = 0.74; p = 0.0001). The GCR ER22/23EK heterozygous polymorphism was associated with an elevated BMI in patients (29.4 versus 24.7 kg/m^2; p = 0.02) and healthy controls (26.3 versus 24.2 kg/m^2; $p < 0.0001$) but with a lower HDLC in patients, than controls. Neither the *Bcl*I nor the N363S polymorphisms were associated with any significant alteration in the metabolic traits examined.

Conclusions

The lower prevalence of Addison's disease in South Africa than Western countries is concerning since patients could be dying undiagnosed. Enhanced awareness of this highly treatable condition is warranted. Autoimmunity predominated in patients mostly of European descent (white), but none of the Asian or black patients had either detectable adrenocortical or 21-hydroxylase autoantibodies necessitating further local studies to understand whether there is a true ethnic predilection for autoimmune Addison's disease. A low threshold is required for screening, intervention and follow-up of all patients for cardiovascular risk factors given the atherogenic profiles of the patients in this study. The supra-

physiological concentrations of salivary cortisol on hydrocortisone replacement should prompt clinicians to screen patients for side-effects. The association between the ER22/23EK polymorphism and elevated BMI in both patients and controls requires confirmation in a large sample. Further local and international studies are warranted to corroborate the findings of this large sub-Saharan study of Addison's disease and to refine the management of this condition.

Chapter 1
Introduction to primary hypoadrenalism and its possible complications

1.1 Introduction

Addison's disease was first described in 1855 by Thomas Addison at Guy's hospital as a disease that caused the symptoms of "general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach, and a peculiar change of colour in the skin, occurring in connection with a diseased condition of the supra-renal capsules".[1] This was an era in which biochemical testing and confirmation were not available. Addison's disease was invariably fatal, but despite the fact that life-saving glucocorticoid (GC) replacement therapy has been available since 1949, multiple challenges remain with respect to its management.[2] At least two famous people are said to have had Addison's disease. A posthumous diagnosis was made in Jane Austen by the symptoms and signs that she had exhibited during her lifetime.[3] President John F Kennedy also suffered from Addison's disease. There has been debate as to whether this illness affected his performance as a president.[4 5]

This overview is intended to review the literature that underpins the work conducted for this thesis. Due to the divergent nature of the fields that it covers, it is inevitable that it is voluminous. The clinical aspects of primary hypoadrenalism are first summarized. This is followed by a description of the anatomy and embryological aspects of adrenal gland development. The aetiology of primary hypoadrenalism is then discussed. As autoimmunity represents an important underlying cause, the pathogenesis of autoimmunity is described, highlighting the normal defences against autoimmunity and why these should fail. As autoimmune primary hypoadrenalism frequently occurs as part of a cluster of autoimmune endocrine conditions, the clinical aspects of autoimmune polyglandular syndromes are explored. The methods available for screening for primary adrenal failure are

then reviewed. In view of patients with Addison's disease not having the same life expectancy as the background population, the possible impact of GCs on lipids and lipoproteins was considered next. This is followed by an appraisal of the methods used for monitoring GC replacement therapy in primary hypoadrenalism and in particular, salivary cortisol in this regard. A review of how the presence of glucocorticoid receptor (GCR) polymorphisms may impact on GC action concludes the literature overview.

1.2 Clinical presentation

Addison's disease may present either with acute or chronic primary adrenal insufficiency, which have substantially different features.

1.2.1 Acute primary adrenal insufficiency

Acute primary adrenal insufficiency is characterised by orthostatic hypotension, agitation, confusion, circulatory collapse, abdominal pain and fever. Acute adrenal decompensation is caused by haemorrhage per se, or rarely by bleeding into metastases of the adrenal glands, often precipitated by coexistent acute infection leading to death if left untreated.[6 7]

1.2.2 Chronic primary adrenal insufficiency

The diagnosis of chronic primary adrenal insufficiency is frequently preceded by a history of prolonged hyperpigmentation, malaise, fatigue, anorexia, weight loss, gastrointestinal disturbance, and joint and back pain. Patients may crave salt and develop unusual food preferences, such as drinking the brine surrounding pickles.[8] Hyperpigmentation is the most frequently encountered sign. It is more easily recognised in the sun-exposed areas of the face, neck and arms, and also occurs on areas that are subject to trauma such as the knees and knuckles. However hyperpigmentation may be more difficult to recognise in darker-skinned races, as the palmar creases and mucous membranes are often normally pigmented.[6] Extensive or progressive hyperpigmentation on any of these sites

should alert clinicians to the possibility of Addison's disease. However, increasing pigmentation of the skin is not diagnostic of primary hypoadrenalism.[9] Scalp hair may also become darker, new naevi may be observed[10] and calcification of the cartilage of the ear may occur.[11]

Primary autoimmune adrenal insufficiency may exist as a component of autoimmune polyglandular syndrome (APS). Vitiligo may be demonstrable in up to 9% of patients with APS1 and 5% of patients with APS2. An unusual manifestation of vitiligo is that scalp hair although hyperpigmented initially, may ultimately lose its pigmentation.[10]

Hyponatraemia, hypoglycemia, hyperkalaemia, unexplained eosinophilia and mild pre-renal dysfunction are together highly suggestive of primary hypoadrenalism.[12] [13] Although the finding of recurrent hypoglycaemia in a type 1 diabetic should alert a clinician to the possibility of Addison's disease, it is an uncommon cause of recurrent hypoglycaemia (1%).[14] [15]

1.3 Anatomy and embryology of the adrenal glands

The adrenal glands are small triangular glands; one located above each kidney. The left adrenal gland is crescent-shaped and the right gland is pyramidal in shape (Figure 1). Both glands are surrounded by firm fibrous capsules that often merge with the perinephric capsules. The adrenal glands are supplied by the phrenic artery, the abdominal aorta and the renal arteries.[16] The veins draining the adrenal glands are the shorter right adrenal and the left longer adrenal veins. Variations of venous drainage and arterial supply are known to occur, and one case of the right middle adrenal artery arising from the right renal artery was recently documented.[17] The major lymph vessels drain the medulla and subcapsular adrenal cortex and culminate in the paraaortic, paracaval and perirenal lymph nodes.[18]

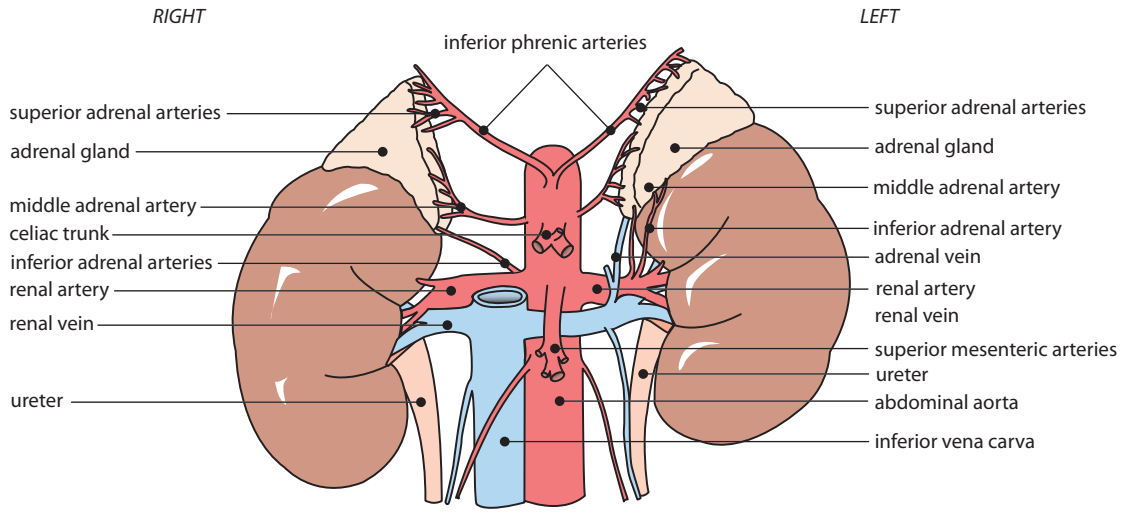

Figure 1: The anatomy of the adrenal glands. The arterial supply, venous drainage and relations of the adrenal glands are shown. Adapted from Human anatomy Martine FH, Timmons MJ, Robert MB et al, 2006 The endocrine system, the adrenal gland Chapter 19, Figure 19.9 page 513. Berriman L Daryl Fox San Francisco Fifth edition.[19]

The adrenal glands have a larger outer cortex and a smaller inner medulla. The glands measure between 3 cm and 4 cm at their longest diameters, have a mass of between 5 to 7g,[20] and have a yellow colour imparted by the rich lipids within the cortices. The adrenal medulla, by contrast, is reddish-brown.[21] In most mammals, the adrenal cortex consists of an outer zona glomerulosa, intervening zona fasciculata and an inner zona reticularis. A narrow population of stem cells surrounds the zona glomerulosa, facilitating progressive development. This population of cells may ultimately give rise to adrenocortical neoplasia.[22]

1.3.1 Embryological development of the adrenal glands

The mesodermal ridge gives rise to both the kidneys and adrenal cortices, which helps to explain their close anatomical proximity. At around the fourth week of gestation, the coelomic epithelial cells and the underlying mesonephric mesenchymal cells migrate from the mesonephros to form the most rudimentary steroid-producing tissue.[23] By day 25 bilateral adrenal primordia develop as cords

of large polyhedral cells of coelomic mesothelium. Primitive sympathetic cells migrate with nerve tracts to form the adrenal medulla. In the seventh week in utero, the paraganglionic cells replicate and differentiate and by the eighth week, discrete foetal gonadal and adrenal tissues are discernible, which then develop as separate entities thereafter (Figure 2). Between the eighth and ninth weeks of gestation, adrenal glands are encapsulated, which results in a distinct organ cranial to the kidney.[23]

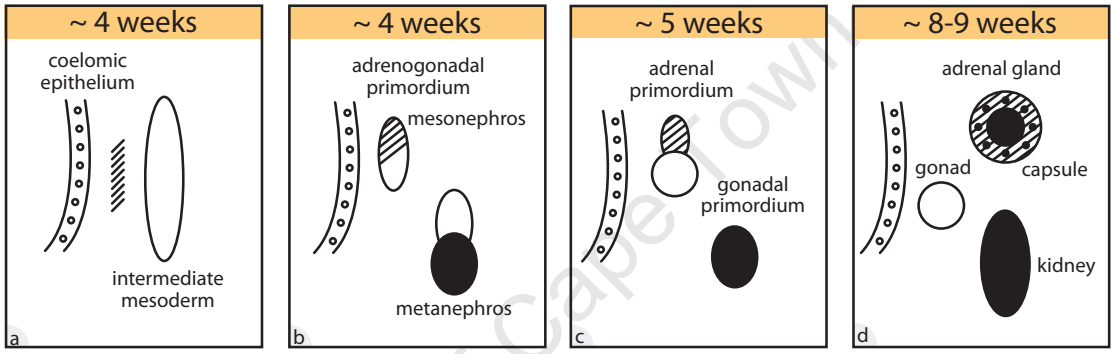

Figure 2: The significant events in the early human adrenal gland development. a) The adrenal cortex develops from the intermediate mesoderm. b) The intermediate mesoderm contains adrenogonadal progenitor cells, which give rise to steroidogenic cells of the adrenal gland and gonads. The cells due to become adrenal tissue migrate retroperitoneally to the upper pole of the mesonephros. c) At about 5 weeks, the adrenal and gonadal primordial develop as separate entities. d) Between 8 and 9 weeks, the adrenal gland becomes encapsulated and already exhibits a foetal and definitive zone. Adapted from Ferraz-de-Souza B, Achermann JC. Disorders of adrenal development. 2008. Endocrine development. 13:19-32[23]

The human foetal adrenal gland comprises two distinct zones; the foetal zone and the definitive zone. The former accounts for 80-90% of the adrenal cortex at term and is responsible for the elaboration of mainly androgens, especially dehydroepiandrosterone (DHEA). The definitive zone is responsible for producing cortisol. A transitional zone exists between these two zones and is capable of GC production in the third trimester.[24] The adrenal cortex development in mice

has a persistent postnatal layer adjacent to the medulla known as the X zone. In many respects, the X zone is similar to that of the foetal adrenal tissue. The development of the adrenal cortices is different in mice and humans as CYP17 is expressed only transiently in the former, explaining why mice adrenal glands produce primarily corticosterone rather than androgens.[22] At birth the significant reduction in adrenal volume is matched by a rapid reduction in the levels of androgens. A specific molecular clock has been proposed to direct the number of cell divisions that facilitates involution of the foetal zone. Foetal pituitary adrenocorticotrophic hormone (ACTH) is responsible for the phenomenal growth of the foetal zone,[25] while insulin-like growth factor (IGF II), fibroblast growth factor and epidermal growth factor mediate growth of the foetal adrenal gland.[26] The development of the vascular network may be facilitated by vascular endothelial growth factor (VEGF) and angiopoietins.[27]

1.3.2 Molecular aspects of adrenal development

The molecular underpinnings of adrenal development are highly complex, rapidly expanding and in the last two years alone (between August 2008 and August 2010) 51 publications were identified in a Pubmed search. The formation of the primitive adrenogonadal primordia (AdP), occurs under the influence of transcription factors empty spiracles2 (Emx2), Lin 11 Islet 1 and Mec-3 homeobox gene 1 (Lim1), Wilms tumour 1 (Wt-1) and wingless-type MMTV integration site family member 4 (Wnt4).[28] Figure 3 demonstrates the various combinations of transcription factors that direct the development of undifferentiated primordial cells to form the adrenal cortex, kidney, bipotential gonad and internal reproductive tract primordia.

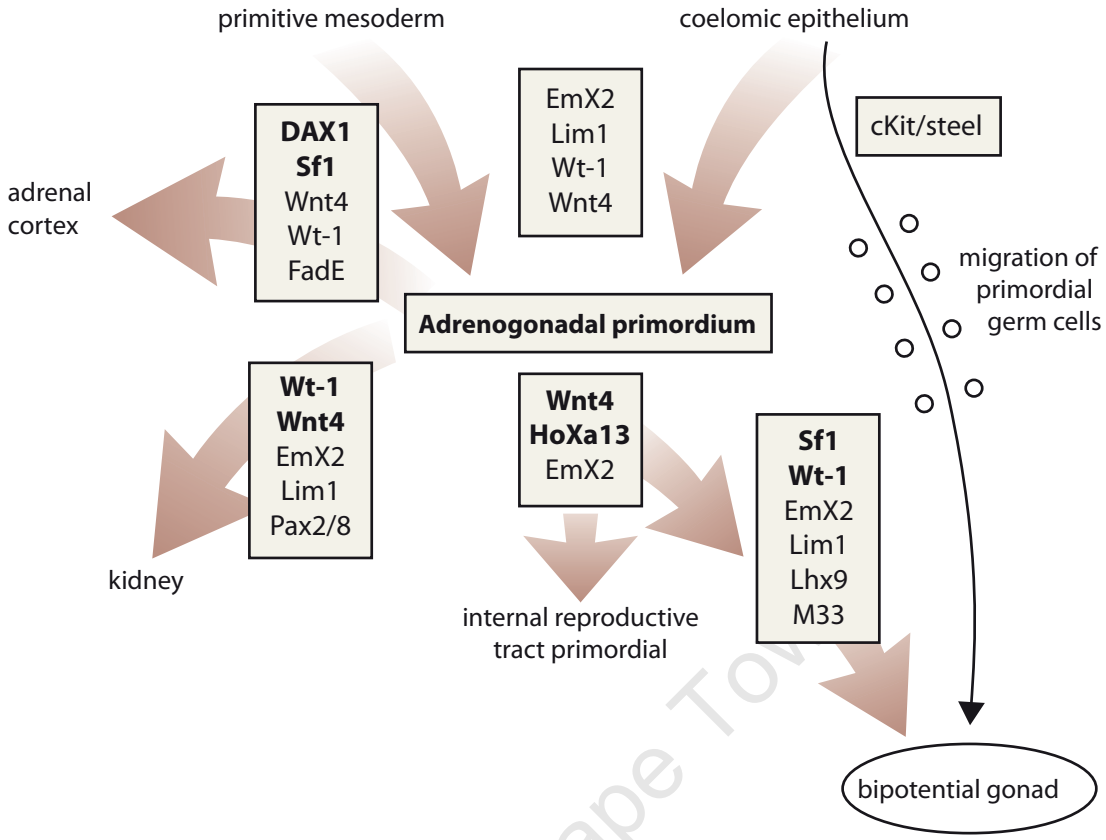

Figure 3: Molecular and genetic aspects in adrenal gland and gonadal development. Adapted from Principles of Molecular Medicine pg 410, Marschall Stevens Runge, Cam Patterson.[28] Transcription factors Emx2, Lim1,Wt-1 and Wnt4 are critical for the development of the adrenogonadal primordium. This forms following amalgamation of primitive mesoderm and coelomic epithelium. Thereafter various combinations of transcription factors will direct the fate of the undifferentiated primordial cells along one or a number of pathways to form the adrenal cortex, kidney, bipotential gonad and internal reproductive tract primordia. Factors highlighted in bold are involved in human development.

Abbreviations:

DAX1 dosage-sensitive-sex-reversal-adrenal hypoplasia congenita locus on the X-chromosome gene 1, Emx2: empty spiracles 2, Hoxa1: homeobox A 1, Lhx9: lim homeobox gene 9, Lim: 1 Lin11 Islet 1 and Mec-3 homeobox gene 1, Sf1: steroidogenic factor 1, Wt-1: Wilms tumour 1, Wnt 4: Wingless-type MMTV integration site family member 4. FadE: foetal zone specific Sf1 enhancer.

The AdP has its origins at the primitive urogenital ridge[29] and its formation is critically dependent on the expression of the nuclear receptor steroidogenic factor 1 (Sf1).[30] Both these primitive organs appear to arise from a single group of cells because they stain immunohistochemically strongly positive for Sf1.[29] The bilateral AdP then divide into the foetal adrenal and the gonadal primordia. Wnt4 is critical for this separation of the AdP.

Foetal zone specific Sf1 enhancer (FadE) is specific for adrenal development, as it is absent in the gonadal primordium.[29] The AdP expresses Wt-1 and Wnt4, which have been found to be crucial in the differentiation of both adrenocortical and gonadal stromal cells.[31 32] The Wnt pathway regulates proliferation, specification of cell fate, stem cell maintenance and differentiation. Wt-1 is responsible for the molecular specification of the adrenal primordia by up-regulation of Sf1. Development of the adrenal primordia is directed through the up-regulation of Sf1 by Wt-1, transcriptional co-activator CREB-binding protein/P 300-interacting transactivator, with ED-rich tail, 2 (Cited2). As the adrenal primordia separate from the AdP, they are subject to the action of a transcription complex containing the homeobox protein [(PKNOX1, homeobox gene 9b and pre-B-cell leukaemia homeobox 1 (Pbx1)] and their principal function is to maintain Sf1 foetal zone expression.[30 33]

It has been suggested that stem cells within the capsule give rise to the definitive cortex in response to a number of morphogenic signals.[33] Simultaneous with the transcriptional cascade, mesenchymal cells that are Sf1 negative coalesce to form a capsule around the foetal cortex (Figure 4). As soon as the capsule is complete, the adult cortex develops in the intervening tissue between the capsule and foetal cortex. Following various mitogenic stimuli, the capsule undergoes symmetrical division, developing another capsule cell, which in turn produces a subcapsular progenitor cell. The capsule and subcapsular population of cells play a critical role in adrenocortical growth and maintenance. These cells are also

receptive to ACTH and angiotensin II signals, which direct the differentiation of these cells to producing steroids.[33]

Sf1/adrenal 4-binding protein (Ad4BP) like other nuclear hormone receptors, has an N-terminal zinc finger DNA-binding domain, a ligand-binding domain and a C-terminal AF-2 activation domain.[34] Sf1 actively promotes a variety of steroidogenic enzymes following ACTH stimulation and is essential not only for providing the subcapsular cells with their ability to proliferate, but is also essential in interacting with other transcription factors.[33]

Sf1 also interacts with transcription factors cyclic AMP response element binding protein and β-catenin. Pbx1 is a downstream mediator of Sf1-dependent proliferation of subcapsular cells. DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region, on chromosome X gene 1) positive together with Sf1 positive progenitor cells are responsible for transiently amplifying non-steroidogenic cells.[33] Investigation of patients with adrenal hypoplasia congenita has revealed that nuclear receptors Sf1, DAX1, and the Pbx1 play pivotal roles in the development of the adrenal cortex. The homeobox genes are thought to induce the Ad4BP/Sf1 expression in the intermediate mesoderm and interact with Pbx1, critical in the early development of adrenal development.[30] FadE functions to fine-tune expression of the Ad4BP/Sf1.[30] The amount of Ad4BP/Sf1 expressed will ultimately determine the size of the mature adrenal gland. Interestingly, DAX1 and Sf1 mutations produce a similar clinical picture of X-linked adrenal hypoplasia congenita, suggesting their interaction. The DAX1 gene product acts as a repressor of Sf1 transactivation, preventing transcription. By contrast, ACTH stimulation inactivates DAX1 and initiates steroidogenesis.[33]

Wnt signalling is crucial in the canonical and planar polarity pathway (Figure 4). In the absence of Wnt ligands, β-catenin is found predominantly in the cell membrane and cellular adherence junctions. Following binding of ligands to Wnt, β-catenin

accumulates in the cytoplasm and nucleus, resulting in the activation of various genes including Sf1 and DAX1, suggesting that β-catenin has an important role to play in adrenal cortex development through proliferation, specification of cell fate, stem cell maintenance and differentiation.[35 36] As Wnt4 is a secreted protein with a critical role in female genital and adrenal gland development, disruption of this protein in a mouse model resulted in abnormal differentiation of the adrenal cortex and sex-reversal.[37] Aberrant migration of adrenocortical cells into the developing gonad was observed in the absence of Wnt4.[38]

Very large quantities of inhibin-α are produced by the foetal adrenal gland, which ensures adrenal specification and unresponsiveness to human chorionic gonadotropin-mediated ovarian development.[33]

All GATA transcription factors are zinc finger transcription factors that bind to specific DNA sequences. GATA-4 has been detected exclusively in the foetal adrenal gland and has been shown to work in concert with Sf1 for adrenocortical development.[22] GATA-4 plays an essential role in upregulating inhibin-α, 17 α-hydroxylase (CYP17) and steroidogenic acute regulatory protein (StAR). Ad4BP/Sf1 is expressed in three zones of the adrenal cortex and is critical for the regulation of the genes encoding the steroid hydroxylases.[39]

Sonic hedgehog (Shh) is a signalling glycoprotein that is known to have a key morphogenic function in organogenesis including limb development.[40] It binds to a receptor of Patched 1 (PTCH1) and Smoothened (SMO), which in turn activates the downstream pathway of glioma associated oncogene homologue (Gli)1, Gli2 and Gli3 transcription factors. Shh is expressed by relatively undifferentiated steroidogenic cells and is involved in the expansion of the progenitor pool in the adrenal capsule. Sf1 positive adrenocortical cells expand the cortex by producing Shh upon the adrenal capsule.[41] The subcapsular cells gain Sf1 expression, but have limited steroidogenic potential. They continue to proliferate and migrate

towards the corticomedullary boundary and mature, acquiring full steroidogenic activity.[33]

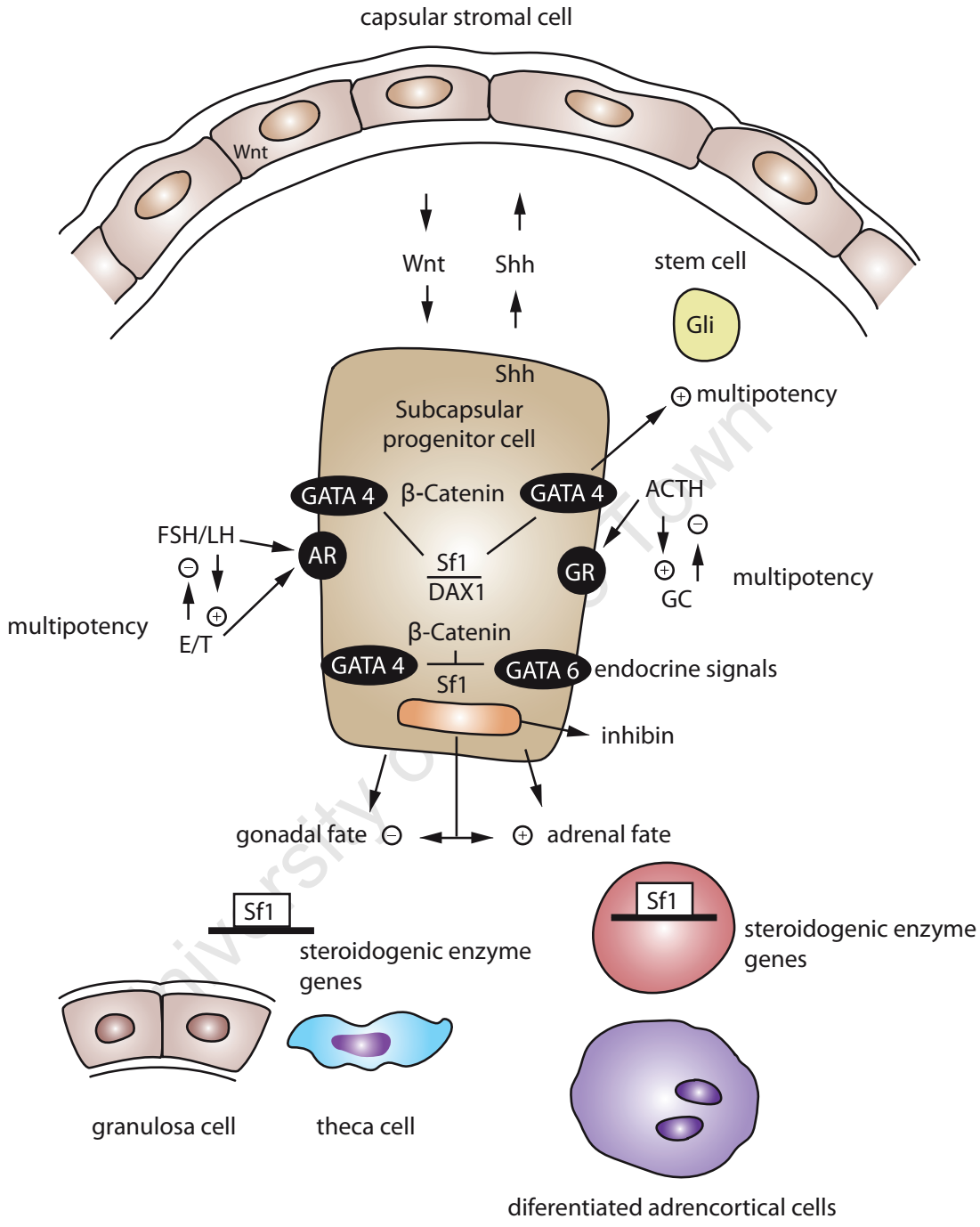

Figure 4: Molecular aspects of adrenal cortical cell development. Adapted from Kim AC, Barlaskar FM, Heaton JH, Else T, In search of adrenocortical stem and progenitor cells 2009 Endocrine Reviews pages 241-263.[33] Stem cells within the capsule give rise to the definitive cortex. Mesenchymal cells negative for Sf1 (steroidogenic factor-1) coalesce to form a capsule around the foetal cortex. Following mitogenic stimuli, the capsule produces another capsule cell, which in turn produces a subcapsular progenitor cell. ACTH and angiotensin II direct the differentiation of these cells to produce steroids. The DAX1 gene product acts as a repressor of Sf1 and prevents

transcription, while ACTH inactivates the former. Sf1 interacts with other transcription factors for adrenocortical development. Wnt signalling is crucial in the canonical and planar polarity pathway. Wnt4 is involved in female genital development. Large quantities of inhibin-α ensure adrenal rather than ovarian development. GATA-4 plays a role in up-regulating inhibin-α, CYP17 and StAR (steroidogenic acute regulatory protein) and is thus critical for up-regulating the genes encoding the steroid hydroxylases. Shh (Sonic hedgehog) is a glycoprotein and has a key morphogenic function. Shh is expressed by relatively undifferentiated steroidogenic cells and expands the progenitor pool in the adrenal capsule. The subcapsular cells gain Sf1 expression, but have limited steroidogenic potential. They continue to proliferate and migrate towards the corticomedullary boundary acquiring full steroidogenic activity. E: Oestrogen; T: testosterone AR: androgen receptor; GR, glucocorticoid receptor; GC: glucocorticoid. FSH: follicle stimulating hormone; LH: luteinising hormone

1.3.3 Foetal production of cortisol and dehydroepiandrosterone (DHEA)

StAR, 11 beta-hydroxylase, 17α-hydroxylase, 3 β-hydroxysteroid dehydrogenase and 21-hydroxylase enzymes have been detected by immunohistochemistry at 50-52 days postconception. The presence of high concentrations of transient nerve growth factor IB-like (NGFI-B) through its regulation of type 2 3 β-hydroxysteroid dehydrogenase, along with early ACTH secretion from the pituitary in the first trimester may be responsible for the early production of cortisol,[42] while epidermal growth factor (EGF) may be responsible for promoting adrenocortical growth.[43] Taken together it appears as though the combination of NGFI-B and ACTH are critical in the early development of a functioning hypothalamic-pituitary adrenal (HPA) axis, but the mechanisms that regulate NGFI-B expression and the precise order in which control occurs remain uncertain.[42] The volume of steroid hormones produced is influenced by the arterial perfusion of the adrenal, which in turn is affected by the prevailing partial pressure of oxygen, endothelin and nitric oxide. ACTH exerts a vasodilator action on the arteries, which supply the adrenal cortex. The zona glomerulosa is able to release epoxyeicosatrienoic acids (EETs) in response to ACTH secretion. These EETs induce vascular smooth muscle relaxation and ultimately lead to growth of the adrenal cortices.[44] Concomitantly the human corticotrophs produce ACTH.[45] DHEA has been detected as early

as the onset of the second trimester. Large volumes are first secreted during the second and third trimesters by the adrenal cortices.[42 46]

1.4 Aetiology of Addison's disease

Although an increasing prevalence of primary adrenal insufficiency has been reported in Western countries over the past four decades, it is unclear whether this constitutes a true rise in prevalence or heightened awareness.[47 48] Our understanding of the aetiology of Addison's disease has evolved over time. In the latter half of the 20th century it has been a decline of Addison's disease attributed to tuberculosis from 33% to 2.6%, while the majority are now thought to be autoimmune in origin.[49] Indeed, it is only since the discovery of specific adrenal autoantibodies that a definitive diagnosis of autoimmune Addison's disease could be made. Autoimmune Addison's disease is the most common form of primary adrenal insufficiency in Western countries, accounting for 68-94% of cases, even though this has sometimes been assessed by adrenal autoantibody assays run in excess of 10 years after the onset of clinical disease.[48-51] In an Italian study, 70% of patients with previously designated idiopathic Addison's disease, with a disease duration of less than 20 years, were positive for adrenal autoantibodies, which is consistent with an autoimmune aetiology.[52] Positive adrenocortical autoantibodies (ACA) and 21-hydroxylase autoantibodies were reported in up to 90% of patients with recent-onset autoimmune Addison's disease, compared with 0.3% among healthy Italian control subjects.[53-55]

Autoimmune Addison's disease may occur as an isolated condition or in association with the APS. In childhood,[6 49] APS1 occurs in association with hypoparathyroidism and mucocutaneous candidiasis and in adulthood APS2 occurs in association with type1 diabetes mellitus (T1DM) and autoimmune thyroid disease.[56]

A wide variety of infections have been ascribed to cause Addison's disease. Of these tuberculosis accounts for the majority, while systemic mycoses for example

histoplasmosis, coccidiomycosis and blastomycosis, occur relatively rarely.[6] In most recent Western series, tuberculosis accounts for 15% of all Addison's disease cases.[55] Adrenal insufficiency secondary to histoplasmosis is extremely rare,[57] but asymptomatic infection of the adrenal glands by histoplasmosis occurs more commonly and should be considered in the differential diagnosis of enlarged adrenal glands.[58 59] Adrenal involvement due to Cryptococcus neoformans, Toxoplasma gondii, Mycobacterium avium-intracellulare and Kaposi's sarcoma, usually in an in immunocompromised state, arise from human immune deficiency virus (HIV) infection.[60] Hyponatraemia may be falsely attributed to gastrointestinal involvement, particularly severe diarrhoea, rather than to hypoadrenalism secondary to HIV infection.[61 62] In one study, frank hypoadrenalism in advanced HIV was reported as remarkably uncommon.[61] End-stage acquired immune deficiency syndrome (AIDS)-associated opportunistic infections, for example cytomegalovirus (CMV) or Mycobacterium avium-intracellulare, may impair adrenal function by direct invasion of the adrenal cortex. CMV may remain latent for many years and become activated in an immunocompromised individual. CMV may affect the retina, oesophagus and the interstitium of the lungs. Thus, these coexisting conditions may suggest that CMV may be the underlying cause for Addison's disease.[63] A single case report has described Addison's disease in a paediatric patient with CMV and HIV-1 co-infection.[64]

The adrenal glands are frequently infiltrated by metastatic and lymphomatous spread from primary carcinomata of the lung, breast, kidney, bladder, pancreas, melanomata and haematological malignancies, and hypoadrenalism can occur.[65] This was recognised as early as 1855 by Addison, who first suggested that adrenal metastases could induce adrenal insufficiency, but the prevalence of hypoadrenalism in this context is remarkably poorly documented.[66] At least 90% of the adrenal glands need to be replaced by tumours in order for primary hypoadrenalism to result. Although it is mandatory to exclude adrenal dysfunction where there is CT evidence for enlarged adrenal glands, normal adrenal function

may persist, despite significantly enlarged adrenal glands.[67-70] Interestingly, many of these patients demonstrate a paradoxical supra-normal cortisol response to ACTH stimulation testing, which is thought to be the result of chronic stress.[71]

Lung cancer is the leading cause of cancer mortality in most countries, with the global incidence increasing by 0.5% per year.[72] In a series of 500 consecutive cancer autopsies, 42% of metastatic lung cancers involved the adrenal glands. This high prevalence of adrenal metastases may reflect the rich sinusoidal blood supply and high local concentration of GCs, which may promote implantation of metastases.[73] There are sparse data on the prevalence of hypoadrenalism in metastatic bronchogenic carcinoma. Redman and colleagues,[74] reported a 33% prevalence of hypoadrenalism in 15 patients with a variety of metastatic carcinomas (including lung, colon, gastric, ovary and an unknown primary) and bilateral adrenal enlargement on CT scan. However, the criteria for hypoadrenalism used (cortisol increment of <150 nmol/L or a peak cortisol concentration <415 nmol/L), do not accord with accepted criteria for primary adrenal insufficiency.[47 75] Lutz and colleagues[76] employed more conventional diagnostic criteria and studied patients with either unilateral (n = 8) or bilateral (n = 9) adrenal metastases, not exclusively bronchogenic, and found no evidence for overt hypoadrenalism.[76]

Several factors may potentially account for under-reporting of hypoadrenalism associated with metastatic carcinoma. Firstly, since the symptoms of adrenal insufficiency are similar to those of extensive metastatic disease, adrenal insufficiency is seldom considered in a patient whose condition is progressively deteriorating with increasing anorexia, malaise and weight loss.[77] Secondly, the treatment of metastatic cancer of the lung with GCs may mask the state of adrenal insufficiency. Thirdly, since the syndrome of inappropriate anti-diuretic hormone (SIADH) is often encountered in small-cell carcinoma, hyponatraemia may be incorrectly attributed to SIADH rather than primary adrenal insufficiency.[78] Additionally, there have been reports of adrenal insufficiency as the sole

manifestation of cancer and of malignancies initially presenting as an Addisonian crisis.[79 80] To address this uncertainty, the prevalence of hypoadrenalism using validated diagnostic criteria in patients with advanced (stage III or IV) bronchogenic carcinoma and were not pre-selected for adrenal metastases, was investigated. In this cohort of 30 subjects, two patients had definitive evidence for adrenal insufficiency, with the peak cortisol of 536 and 545 nmol/L, associated with a raised plasma ACTH concentration of 131.4 pmol/L and 10.5 pmol/L, respectively (normal 2.2-10.0 pmol/L) following a 250 μg ACTH stimulation test. The overall prevalence of adrenal insufficiency was 6.7% in the series (95% confidence interval 0.8%-22.1%),[7] which was in contrast to the 33 % reported by Redman et al.[74]

Adrenoleukodystrophy (ALD) is a rare X-linked condition (1:20 000 males) characterised by a deficiency of peroxisomal membrane ALD protein, which transports activated acyl-CoA derivates into the peroxisomes, where they are shortened by beta-oxidation. The ALD protein, which is similar to the adenosine triphosphate (A.T.P.)-binding cassette transporter super family of proteins, is encoded by the ALD gene mapped to Xq28.[81] This deficiency results in accumulation of very long chain fatty acids (VLCFAs) in the blood, adrenal gland, brain, testis, and liver.[82] The ensuing demyelination may evoke an autoimmune reaction.[83] The adrenal glands may sustain damage by VLCFA accumulation, inducing cell membrane microviscosity and subsequent alterations in ACTH action.[84]

The presentation of ALD may vary widely, with six distinct described types ranging in decreasing order of severity down to asymptomatic individuals. The childhood cerebral type is the most devastating form. It occurs in 31-35% of patients with ALD, and is characterised by adrenal insufficiency and progressive relentless neurological dysfunction, often presenting with cognitive and gait disturbances, evolving to a vegetative state within 2-4 years.[85 86] The adolescent and adult

cerebral form occurs in 6-12% of patients with ALD and resembles the child-type in which spinal cord, peripheral nerve and psychiatric symptoms predominate. However, its progression is far slower than the childhood cerebral form.[85-87] Adrenomyeloneuropathy occurs in 40-46% of patients with ALD and typically, the age of onset is between 20 years and 40 years of age. It has a reduced propensity to affect cortical function, and a greater tendency to affect the long ascending and descending tracts of the spinal cord, inducing inter alia urinary and erectile dysfunction along with hypoadrenalism.[88] Approximately 10-20% of X-linked ALD patients have primary adrenal insufficiency without neurological involvement and a proportion may be entirely asymptomatic, despite very high levels of VLCFA. Female carriers may have neurological involvement due to non-random X inactivation, with a similar clinical picture to adrenomyeloneuropathy, but a slower rate of progression and rarely, adrenal involvement.[85] A proportion of patients with Addison's disease due to ALD without neurological damage exhibit increased VLCFA and their brothers also demonstrate increased VLCFA.[82] Although dietary restriction of VLCFA, particularly hexacosanoic acid (C26:0) may normalise plasma VLCFA, it does not produce a significant clinical improvement and the ongoing deterioration may be accounted for by an autoimmune process.[83]

Sarcoidosis has previously been suggested as a cause of Addison's disease. However, some reports suggest that the sarcoidosis may have been coincidental, as the Addison's disease coexisted with another autoimmune gland failure compatible with Schmidt's syndrome.[89]

The triple A or Allgrove's syndrome is an autosomal recessive disorder, characterized by the triad of achalasia cardia, alacrima and ACTH-resistant adrenocortical insufficiency. It is also known as the achalasia-Addisonianism-alacrima syndrome (AAAS) and results from mutations on chromosome 12q13.[90] The antiphospholipid syndrome may result in primary hypoadrenalism, either due to adrenal haemorrhage and subsequent haemorrhagic destruction following

vascular occlusion of the adrenal vessels, or secondary to anticoagulant therapy in the presence of antiphospholipid antibodies. Autoimmune mechanisms could also be causative in association with the antiphospholipid syndrome.[91] Intra-adrenal haemorrhage may hinder normal steroid hormone production within the adrenal cortex, infrequently resulting from long-term anti-coagulation therapy.[6] Haemorrhaging of the adrenal cortices has been demonstrated following prophylaxis with anti-coagulation for joint replacements.[92] It has been postulated that a stressed adrenal gland, that is significant ACTH stimulation, may be more predisposed to haemorrhage if anticoagulation is concurrently administered.[93]

A rare cause of primary adrenal insufficiency is Erdheim-Chester disease, which is a non-Langerhans form of histiocytosis, characterised by xanthomatous infiltration of tissues with foamy histiocytes. The pituitary gland is the most common gland to be affected and primary hypoadrenalism has been documented rarely. In the largest case series (n = 22) of Erdheim-Chester disease, adrenal enlargement was detected in seven cases (31.8%), but only one patient had adrenal insufficiency.[94]

Mutations in transcription factors responsible for normal adrenal gland development have been found to induce a familial syndrome of congenital adrenal hypoplasia. These include mutations of any of the following: DAX1 [dosage-sensitive sex reversal, adrenal hypoplasia congenital (AHC), critical region on the X chromosome, gene-1, NR0B1/AHC] and steroidogenic factor-1 (Sf1, NR5A1 and Ad4BP.[95] [96] Males with congenital adrenal hypoplasia usually present in infancy or early childhood with salt-losing primary adrenal failure, recognised by profound hyponatraemia, global GC deficiency in infancy and arrested puberty because of associated hypogonadotropic hypogonadism.[96] Duplication of the NR0B1 induces a [46], XY disorder of sex development resulting in XY “sex reversal” females.[97]

The Smith-Lemli-Opitz syndrome is a genetic disorder resulting from 7-dehydrocholesterol reductase mutations in the gene DHCR7 producing growth failure, mental retardation and craniofacial malformations. Moreover some mitochondrial DNA deletions resulting in the Kearn's Sayre syndrome may cause external ophthalmoplegia, retinal degeneration, cardiac conduction defects and other endocrinopathies.[2] Several drugs have been implicated in causing adrenal insufficiency with glucocorticoid treatment being the most common and exposing it on withdrawal. Other drugs include anticoagulants which may induce adrenal haemorrhage, while aminoglutethamide, ketoconazole, fluconazole and etomidate are able to inhibit specific enzymes of cortisol synthesis. Moreover, phenobarbital, rifampicin and phenytoin inter alia have resulted in activation of cortisol metabolism.[98]

1.4.1 Pathogenesis of autoimmunity

The cardinal function of the immune system is to protect an organism from infection. Vertebrates have developed a large range of T-cell and B-cell receptors that alert an organism to infectious agents. Once an offending agent has been identified, there are multiple mechanisms with which an organism can defend itself. The particular mechanism it selects depends on the site and the type of prevailing infectious agent. For example, if the offending agent is in the blood circulation, antibodies are likely to provide protection.[99] Separate immune defence mechanisms operate in humans. The most rudimentary system or innate immunity functions to defend the organism against oxidative stress, but must discriminate between foreign and self. The most sophisticated immune defence system relies on clonal selection of specific antibodies.[100] Since natural killer (NK) cells share characteristics of both innate and adaptive immunity including harbouring memory of a previous exposure to specific antigen, execution of a brisk response to a pathogen and secretion of interferon-γ in response to a cognate ligand, it could be considered to act as a bridge between innate and adaptive immune systems.[101] A second population of T-cells expresses a γ-δ T-cell receptor.

Although a relatively small proportion exists in blood, the remainder represents a formidable subset of intraepithelial lymphocytes, capable of recognising inter alia small bacterial phosphoantigens. Some of the γ-δ T-cells require induction prior to their involvement in effector function, and this prepared state may represent innate immunity. As their responses are both compatible with innate- and antigen-specific, they also have the ability to provide intermediate protection between innate and adaptive immunity.[102]

Although diversity is ensured by multiple rearrangements of genes, there is a risk of producing antibodies or T-cell clones that react with self components of the host organism. Enormous diversity of the T-cell receptor functions is achieved through the random rearrangements of both α- and β- genes. These rearranged segments of genes are ultimately spliced together, generating functional mRNA, and in turn, multiple permutations of α- and β- protein chains.[103] Immunoglobulins are produced by B-cell lines, which originate in the bone marrow and then reside in either the spleen or peripheral lymph glands. As in the case of T-cells, multiple genes are responsible for the diverse repertoire of antibodies. The κ and γ genes encode light chains, while a single gene family is responsible for encoding heavy chains. The variable region (V) is responsible for binding directly with the antigen.[104]

Elimination of T-cells that are directed at the self occurs in the thymus and is referred to as negative selection.[105] Certain subsets of T-cells directed at self may escape elimination, due to some autoantigens not being detected in the thymus.[105] [106] Autoreactive B-lymphocyte elimination occurs in the bone marrow.[107] Central and peripheral tolerance ensures that immune cells react intensively with foreign antigens, but weakly with self antigens. Autoreactive cells not eliminated by either of these two mechanisms may induce autoimmunity.

1.4.2 Recognition of a foreign or harmful antigen

T-cell lymphocyte precursor cells originate in the bone marrow and migrate to

the thymus where they evolve into mature T-cells. The thymus facilitates the production of various T-cell receptors. Early T-cells differentiate into either CD4 or CD8 cells.[108] The vast majority of mature T-cells are tolerant to self antigens. T-cells react to foreign antigens that are bound to the major-histocompatibility complex (MHC).[109] T-helper cells are stimulated by antigen-presenting cells (APCs), such as macrophages and dendritic cells. Various chemokines follow initial contact with an antigen.[110] T-helper cells also stimulate B-lymphocytes to evolve into immunoglobulin-producing plasma cells. Mature B-lymphocytes are able to migrate from the bone marrow to peripheral lymphoid tissue. The primary follicles within the lymphoid tissue are dedicated areas where class switching may occur. This is a process where B-lymphocytes, in a heightened state of stimulation can change their immunoglobulin class production.[111] Most B-lymphocytes may become resting cells and serve as memory cells, which are fundamental in ensuring a class switch of immunoglobulins with re-stimulation by the same antigen.[112]

T-cells, but not B-cells require the antigen to be bound to the MHC, which is a series of over [150], interconnected genes with a critical role in the presentation of antigens.[113] MHC is divided into three complexes. Class I and II are responsible for antigen presentation[114] and class III MHC proteins will be discussed below. Foreign antigens or proteins are fragmented within the cells and part of the protein is then displayed in the cell membranes of the MHC. Class I MHC proteins for example, HLA-A, HLA-B and HLA-C are ubiquitous, are found on virtually all cells and have the ability to present peptides, derived from the cell cytoplasm, to CD8 T cells. This is sometimes referred to as the cytosolic or endogenous pathway.[115] T-cells are also designed to destroy endogenous antigens, which are presented through the class I MHC. Viruses or malignant cells, which present abnormal antigens through the former mechanism, may be eliminated by lysis.[116] NK cells are not HLA-A restricted. They produce cytokines, such as interferon gamma (IFN-γ) and are able to eliminate infected or malignant cells, which do not

express HLA class I molecules. Some viruses escape CD 8+ class I expression. Genes encode HLA-A, B, C and CD1 regions.[117] MHC class Ib protein HLA-E has a specialised function that permits cell recognition by NK cells, but it also has the ability to interact with the T-cell receptor.[118]

By contrast, MHC class II molecules are found only on specialised cell types that function as APCs, including macrophages, dendritic cells, Langerhans cells, activated T-cells and B-cells coded by HLA-DP, HLA-DQ and HLA-DR loci.[119] The antigens or proteins presented on class II molecules are derived from extracellular proteins, lipids and polysaccharide entities. The class II MHC has the ability to present peptides from the extracellular and intravascular spaces to CD4 T-cells.[120] The DQ locus consists of two closely related genes, the DQA1 and DQB1, which are responsible for two separate glycoproteins; α and β respectively. Allelic variation of the HLA-DQB1 is of interest, as it modifies the peptide-bound cleft, responsible for presentation of antigens, eliciting a powerful T-cell response.[121]

CD1 protein is also an APC molecule of phospholipid antigens, which permits interaction with the T-cell receptor.[122] The CD1 pathway is a unique one for presentation of lipids and glycolipid antigens by CD1 molecules to specific CD1-restricted T-cells, such as NK. CD1 are “non-classical“ HLA class I molecules with limited diversity. CD1-restricted T-cells perform effector and helper activity. Principally, they interact with macrophages, dendritic cells, NK cells, B- and T-cells, contributing to both the innate and adaptive responses.[119]

The class III region spans about 700 genes and encodes for tumour necrosis factor α and β (TNF-α and β), as well as factors related to complement. This region encodes genes that can escalate the inflammatory and immunological response.[123] The enzyme 21-hydroxylase is also encoded by the class III region of the MHC. [124]

In order for T-cells to become activated, APCs must present an antigen within the MHC and it should be followed by interaction of co-stimulatory molecules. These two signals result in proliferation of T-cells, interleukin-2 (IL-2) production and the elaboration of the product of the bcl-2 proto-oncogene (Bcl-xL), which is an anti-apoptotic protein.[125] [126] T-cells may be activated by T-helper cells expressing CD4 surface proteins, which in turn stimulate cytotoxic T-cell production. They do this by binding of CD40 on APCs with CD154 found on T-helper cells, permitting direct stimulation of T-cytotoxic cells by APCs.[127] [128] Another molecule found on T-cells is the CD28 homolog cytotoxic T-lymphocyte antigen (CTLA-4 or CD152), which functions to suppress T-cell activation and may play a role in the susceptibility to various autoimmune diseases.[129]

Similar to T-cells, B-cells also require at least two signals to become activated. Antigens may stimulate B-cell production directly and T-helper cells are also involved in stimulating B-cell production through the interaction of CD154 on T-helper cells with CD40 on B-cells.[130] This engagement is a powerful stimulus for cytokine production by T-helper cells, which is essential for isotype switching and formation of germinal centres in lymphoid tissues. When B-cells encounter an immunoglobulin, internal B-cell mechanisms are activated and the immunoglobulin serves as an antigen receptor. Subsequently, the degraded protein is transmitted onto the cell surface, which is presented within the MHC II molecule and permits recognition by cytotoxic T-cells. These degraded proteins have the ability to stimulate B-cells, which in turn are able to produce appropriate antibodies.[131] Adaptive immunity is programmed to identify specific molecules and relies on a large number of receptors. As soon as T-cells recognise foreign antigens, a series of adaptive responses are directed at these antigens. Adaptive responses can usually take from days to decades to initiate, and require a combination of B- and T-cells. The armamentarium used to eliminate the foreign antigen includes antibodies; cytotoxic T-cells (CTL); classical complement activation; antibody-dependent cell-mediated cytotoxicity; cytokines and chemokines.[132] It

is crucial that the immune response is highly specific against foreign antigens and not against self antigens. Under normal physiological conditions, there are a number of checks and balances designed to eliminate cells aimed at self. Under most circumstances, these processes preclude the evolution of an autoimmune process.

1.4.3 Prevention of immune responses against self

1.4.3.1 T-cell tolerance

An active state called tolerance, is designed to prevent an autoimmune reaction against self antigens.[133] As previously discussed, cell receptors and antibodies are subject to hypermutation. These random changes may inadvertently result in components of the immune system, directed at self, producing an autoimmune response.[134] T-helper cells mature in the thymus and during development are exposed to a vast variety of self antigens.[135] Clonal deletion is the active process in which maturing T-cells are eliminated if they bind to any of the self antigens, provided that the self antigen is presented within the MHC. The vast majority of developing T-cells undergo apoptosis within the thymus. The remaining mature T-cells that are not eliminated by clonal deletion leave the thymus gland tolerant of self antigens. This active process is often referred to as central T-cell tolerance.[136] Those maturing T-cells (pre-T-cells) that are positively selected as a consequence of their low avidity to MHC-self peptide complexes, increase expression of T-cell receptors and express either as CD4 or CD8. T-cells are also eliminated if they have a high avidity for the MHC-presented self peptide. This process of negative selection may be achieved through apoptosis, tumour necrosis factor receptor, Fas-associated death domain (FADD), or through activation of the Bcl-2 family members, with consequent release of Caspase 9.[137] The factors that determine the low versus high affinity to T-cell receptors are still unknown. T-cells are also activated through the co-stimulatory pathway CD28 and CD80/CD86 on APCs. Models of autoimmune disease have been successful in demonstrating the need

for these important steps as blocking these co-stimulatory pathways abolishes the autoimmune processes.[138] As negative selection of auto-reactive T-cells is imperfect, autoimmunity may result. Anergy is the mechanism by which peripheral tolerance results from the lack of responsiveness to a self antigen. Regulatory T-cells in the form of suppressor T-cells will also function to maintain peripheral tolerance.[139] Auto-reactive T-cells may be eliminated by clonal deletion, which is a modified form of apoptosis in which auto-reactive cells may have escaped elimination.[140] Self antigens may protect against autoimmunity by inducing ignorance to which T-cells do not respond.[141] A shift from a Th1 response to a Th2 response may also suppress auto-reactive inflammatory T-cells, as Th1 responses predominate in the context of autoimmunity.[142] Peripheral tolerance may also be achieved through T-cell active suppression, but the precise mechanism of this is still under review. Natural (innate) or adaptive T-regulatory cells that can suppress via direct cell-to-cell contact, or via release of immune suppressive cytokines IL-10 or TGFβ, have been described. A deficiency in the number of T-regulatory cells or function has been described, as playing an important pathogenic role in various autoimmune diseases, including T1DM. Autoimmunity may thus be prevented if T-regulatory cells successfully antagonise the self-reactive response. However, autoimmunity may occur if the T-regulatory cell response is overwhelmed through inherent defects in the number of regulatory cells, function or persistent chronic inflammation and consequent negative effect on function. Self reactive T-cells may also acquire resistance to the effect of T-regulatory cell mediated control.[143] A novel pro-inflammatory T-cell subset (Th17) producing IL-17, which is a family of cytokines that include IL-17A IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, stimulates neutrophils to the site of inflammation and produce antimicrobial proteins, specifically against Candida and Staphylococcus species. A pathogenic role for Th17 has been suggested in autoimmune animal experiments such as collagen induced arthritis and in human autoimmune diseases, for example rheumatoid arthritis, psoriasis, multiple sclerosis and uveitis. Enhanced Th17 cell expansion, at the expense of T-regulatory cells, may promote autoimmunity.[144]

The role of Th17 in autoimmune Addison's disease is still speculative and further research is required to establish this definitively.[145]

1.4.3.2 B-cell tolerance

The bone marrow is the active site, where central B-cell tolerance is instituted. The pre-B-cells are able to rearrange B-cell receptors in a specific sequence, beginning with heavy chain immunoglobulin gene rearrangement followed by rearrangement of the light-chain. Deletions of clones of immature B-cells, which bind to self antigens, may also be achieved through apoptosis. Editing is the process by which immature auto-reactive B-cells escape apoptosis by undergoing further rearrangement of the light and heavy chains.[146] B-cell tolerance is a vital link in the chain in the armamentarium against autoimmunity. In the absence of stimulation by an antigen, mature B-cells are eliminated by activated T-cells through the Fas-Fas ligand and CD40-CD154 interactions.[147]

1.4.3.3 Pathophysiology of autoimmunity

It is generally assumed that autoimmunity is initiated by activation of the CD4 helper T-cells, which in turn react with a specific autoantigen. An external microbial antigen may induce molecular mimicry due to similarity between this antigen and an autoantigen.[148] Lipopolysaccharides, derived from infectious microbial agents, act as an adjuvant to immune responses. This is accomplished by their binding toll-like receptors to macrophages or dendritic cells, in order to enhance natural immunity and inflammatory cytokine production.[149]

The adaptive immune system makes the assumption that all exogenous antigens are potentially foreign and harmful. It uses specific surface receptors to discriminate between self and foreign antigens.[150] Impairment of the Fas-Fas ligand interaction results in failed apoptosis and consequent autoimmunity.[151] Autoimmunity is often the consequence of failed active suppression, predominant Th1 response and impairment of B-cell tolerance. Genetic factors have long

been considered important in the pathogenesis of autoimmunity[152] especially the MHC.[153] A third of T-helper cells is constituted by a subset of T-helper 17 cells (Th17) and appear to be involved in the development of autoimmune diseases. Th17 cells offer dichotomous immunological function of either protection or immunopathogenesis. T-regulatory cells have an important role in defending against autoimmunity, through the release of IL-10 and transforming growth factor β (TGFβ). It is interesting that TGFβ also permits expansion of T-regulatory cells. There is a reciprocal relationship between T-regulatory cells and Th17.[154] Impaired control by T-regulatory cells on Th17 has been implicated in the evolution of autoimmunity.[155]

Evolution of T1DM is strongly influenced by environmental factors as well as genetic factors. For example, 90% of patients with T1DM harbour a particular DQ B1 allele of MHC. Certain HLA alleles may even offer protection against autoimmunity. For example DQw1.2 is associated with a lower incidence of T1DM.[156] There is overwhelming evidence for DQ alleles being the most important, but not the only markers of susceptibility to autoimmune disease[157] through their ability to alter the conformation of the antigen binding site.[156] Environment also plays an important role in the pathogenesis of autoimmunity, as evidenced by the investigation of T1DM monozygotic twins, which demonstrates concordance of less than 50%.[158] Coxsackie B-virus, avoiding breast-feeding, introduction of certain foods, birth weight and maternal islet autoimmunity are thought to confer the highest risk for T1DM.[159] Epidemiological evidence supports a link between vinyl chloride, silica, exposure to certain metals, polycyclic hydrocarbons, mycotoxins and autoimmunity, through molecular mimicry, alteration of lymphocyte signalling and interference in the development of tolerance to self antigens.[160] Autoimmunity can only result when naive T-cells with auto-reactive potential are exposed to exogenous peptides. T-helper cells stimulate B-cells which in turn produce antibodies and result in tissue damage through the complement pathway. On the other hand, cytotoxic T-lymphocytes may direct the attack at mimicked antigens

presented via the MHC class I molecule.[161]

Data from a variety of sources have ensured that Addison's disease fulfils the criteria of an autoimmune condition. This includes the initial discovery of ACA in 1957,[162] the diffuse mononuclear cell infiltration of the adrenal glands, along with atrophy of the adrenal glands, the presence of a cell-mediated immune reaction[163] and induction of Addison's disease in animals, using cortex extracts and various steroid enzymes as self antigens, presented in association with the MHC. Despite attempts to induce Addison's disease in animals by using autoantigens or steroid enzymes, these have been unsuccessful as they have differed from human disease, in that these failed to induce diffuse atrophy of the adrenal cortices and no regeneration nodules were reported.[164] Additionally, there has been lack of biochemical confirmation of adrenal insufficiency following adrenal antigens.[165]

1.5 Pathogenesis of autoimmune Addison's disease

In non-APS1, certain HLA genes are required for the development of autoimmune Addison's disease, but it also requires the presence of certain environmental triggers. Activated T-lymphocytes are programmed to discern adrenocortical antigens presented in MHC class II molecules. T-helper lymphocytes induce cytotoxic T- and B-lymphocytes by secreting interleukin-2 (IL-2) and other lymphokines, which in turn result in ACA and 21-hydroxylase autoantibody production. T-cell mediated destruction of the adrenal glands is identified by the presence of the latter antibodies in serum.[49]

1.5.1 Autoimmune Addison's disease

Autoimmune Addison's disease may occur as part of the APS or alone.[166] In addition to ACA, several autoantibodies have been detected as being associated with Addison's disease due to the dysregulation of antibody production.[167] It is therefore not surprising that several autoimmune conditions can coexist with

autoimmune Addison's disease. Steroid-cell producing autoantibodies (StCA) and 17 α-hydroxylase autoantibodies were found, for example, in association with the development of coexistent Addison's disease and hypergonadotrophic hypogonadism.[168] Although the presence of circulating adrenal autoantibodies suggests an autoimmune aetiology, occasionally autoantibodies have been found in patients with tuberculous adrenalitis, especially in low titres.[54 169] The levels of autoantibodies fluctuate in concert with disease activity and remit during signs of biochemical remission of adrenal dysfunction. Thus, the reliability of adrenal autoantibody assessment may be influenced by the time elapsed since the diagnosis of hypoadrenalism.[170-172]

1.5.1.1 General statements with respect to autoimmune conditions

A greater frequency of autoimmune conditions occurs in women and in association with the presence of certain human leukocyte antigen (HLA) alleles.[173] The targets in autoimmune endocrinopathies are sometimes an enzyme or cell surface receptor (Table 1). A number of molecular biological factors facilitating an autoimmune process in Addison's disease are likely; such as molecular mimicry, cytotoxic T-lymphocyte antigen-4 (CTLA-4) polymorphisms and aberrant induction of T-cell help. Polymorphisms in the 3'-promoter region and 5'-untranslated region of CTLA-4 predispose susceptible individuals to type IA diabetes mellitus.[174]

Table 1: Confirmed and suspected autoantigens found in autoimmune endocrine conditions frequently associated with autoimmune Addison's disease.[175]

Disease	Confirmed Autoantigens	Putative Autoantigens
Addison's disease	P450c21	P450c17, P450 scc
Hashimoto thyroiditis	Thyroid peroxidase Thyroglobulin	
Graves' disease	Thyrotropin receptor	
Diabetes	Insulin GAD 65, IA-2 ZnT8	Pro-insulin Carboxypeptidase H ICA69
Premature gonadal failure		P450 c17 P450 scc
Pernicious anaema	H+/K+ATP ase Intrinsic factor	
Myasthenia gravis	Acetylcholine receptor-α-chain	
Vitiligo		Tyrosinase Tyrosinase related protein-2
Coeliac disease	Endomyseum	Reticulin Gliadin, Transglutaminase
Hypoparathyroidism	NALP	

Abbreviations:

P450c21: 21-hydroxylase autoantibodies

P450c17: 17 alpha hydroxylase autoantibodies

P450 scc: P450 side-chain cleavage autoantibodies

ICA69: islet-cell autoantibodies

3BHSD: 3-β -hydroxysteroid dehydrogenase

IA-2 insulinoma antibodies 2

GAD 65: glutamic acid decarboxylase 65

Adapted from Paediatric Endocrinology 2008 M Sperling, WB Sanders Philadelphia Autoimmune Polyglandular Syndromes Haller MJ, Winter W E, Schatz DA, page 770-787.[175]

1.5.1.2 Cytotoxic T-lymphocyte antigen-4 (CTLA-4)

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) functions as a co-stimulatory molecule, but it also has an important inhibitory action on T-cell activation.[176] Two sequential steps are required for activation of T cells. The first is the presentation of a foreign antigen by HLA class II molecules on the APCs to the T-cell receptor-CD3 complex. A co-stimulatory signal is required between CD28 on T-cells and the CD80/86 on APCs. CTLA-4, which is a homolog of CD28, also binds CD80/86, but with considerably greater affinity than CD28, which inhibits T-cell activation. Figure 5 demonstrates the known actions of CTLA-4.[177] A CTLA-4 polymorphism has been thought to confer susceptibility to autoimmune Addison's disease, particularly in association with APS.[178] An analysis of CTLA-4 polymorphisms, in both an Italian cohort and a meta-analysis of European Addison's subjects, indicates an association of CTLA-4+49 with proven autoimmune Addison's disease, independent of HLA class II genes.[179] It has been suggested that this polymorphism may reduce circulating levels of CTLA-4, with consequent T-cell activation.[179]

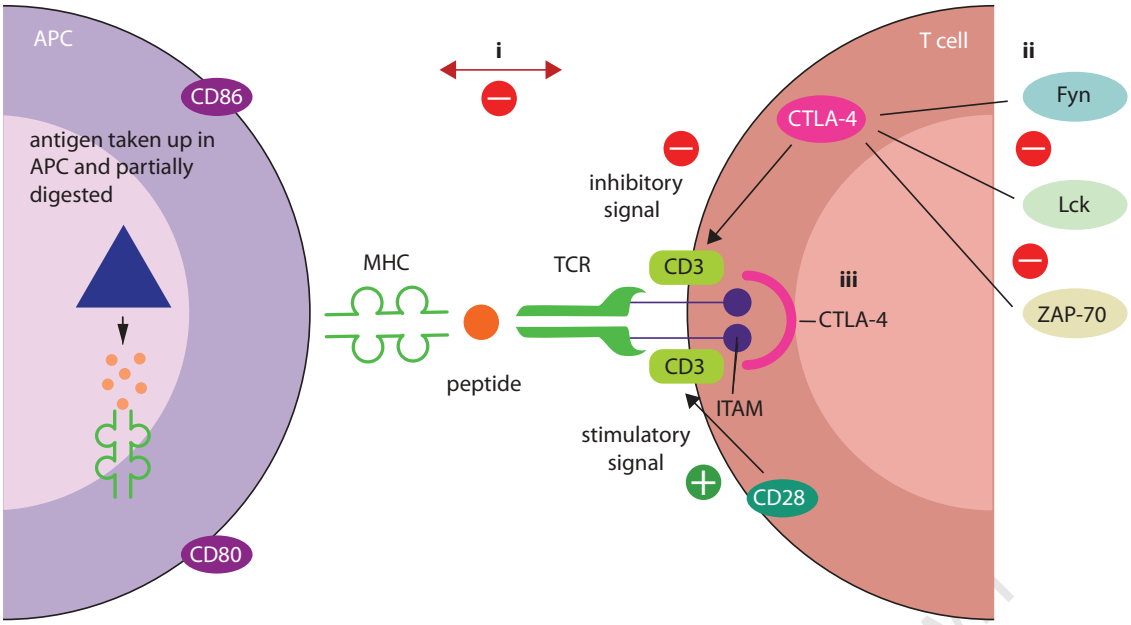

Figure 5: The inhibitory action of CTLA-4 on T-cell activation. (i) CTLA-4 likely competes with CD28 for CD80/86 ligands, inhibiting the co-stimulatory effect of CD28. (ii) CTLA-4 may have an inhibitory action on Lck, Fyn and ZAP-70 protein kinases, which are important in phosphorylating tyrosine residues of the TCR-CD3 complex. (iii) CTLA-4 may interact with TCR-CD3 complex at the immunological synapse to disrupt T-cell activation by binding and blocking the immunoreceptor tyrosine-based activation motif (ITAM).

Abbreviations:
APC: antigen presenting cell
CTLA-4: cytotoxic T-cell associated 4
MHC: major histocompatibility complex
TCR:T-cell receptor
Adapted from HLA, CTLA-4 and PTPN22: the shared genetic master-key to autoimmunity? Brand O, Gough S and Heward J Expert Reviews in Molecular Medicine 2005, volume 7 page 1-15.[177]

1.5.2 Autoantibodies in autoimmune polyglandular syndromes (APS)

Antibodies in certain autoimmune conditions, for example Graves' disease or myasthenia gravis are directly pathogenic, while islet cell cytoplasmic (ICA) and glutamic acid decarboxylase 65 (GAD65) autoantibodies in T1DM are not necessarily so.[180] Nevertheless, demonstration of these autoantibodies in the asymptomatic individual has important predictive value, as it establishes increased risk for the development of clinical disease later in life.[181]

1.5.2.1 Positive autoimmune markers for Addison's disease

Previous studies have shown both 21-hydroxylase autoantibodies and ACA are found in autoimmune Addison's disease, and in the majority their presence is an excellent discriminator of autoimmune versus non-autoimmune Addison's disease. Yet occasionally, they may be present in very low titres in non-autoimmune Addison's disease, in particular unequivocal post tuberculous Addison's disease.[54 169 170 182] Several studies have demonstrated excellent concordance between these autoantibodies, which remain detectable for at least two years after the onset of autoimmune Addison's disease.[49]

1.6 Autoantibodies in Addison's disease

The various autoantibodies found in association with Addison's disease will be discussed below. While the predominant expression of IgG1 isotypes suggests a Th1 response, a small subset of patients with Addison's disease demonstrate IgG4 predominance suggesting a Th2 response in the latter small group.

1.6.1 Adrenocortical autoantibodies (ACA)

Seminal work by Anderson et al in 1957, using the indirect fluorescent antibody technique, recognised that the association of Hashimoto's thyroiditis and Addison's disease produced an antibody to adrenal tissue.[162] ACA are specific to the adrenal glands and capable of binding to the three layers of the adrenal cortex. Immunofluorescence produces a homogenous staining pattern of the cytoplasm.[49] ACA have a positive predictive value for the development of autoimmune Addison's disease, which is greater in children than in adults.[183] In one study, a single patient with adrenal insufficiency related to adrenal tuberculosis demonstrated a positive complement fixation test, but failed to stain positive for the adrenocortical secretory cells by immunofluorescence. This suggested a false positive complement fixation test.[182] ACA are detectable 15 years after the onset of Addison's disease, but the frequency with which they are detected is less than

20%.[172]

1.6.2 Steroid-cell producing autoantibodies (StCA)

Steroid-cell producing autoantibodies (StCA) are not specific to the adrenal glands. They have been found in the presence Addison's disease, often in association with hypergonadotrophic hypogonadism, particularly in women, but they have also been found in men.[49] StCA are polyclonal IgG, producing an identical immunofluorescent pattern to ACA and have been found to be positive in 28% of unselected cases of autoimmune Addison's disease.[49 184] It has been suggested that StCA are useful in detecting autoimmune Addison's disease patients at risk for subsequent primary ovarian insufficieny (POI).[185]

1.6.3 17 α-hydroxylase autoantibodies

17 α-hydroxylase autoantibodies are detected using S-labelled recombinant antigens.53 They react with 17 α-hydroxylase enzyme and are also not specific to the adrenal glands, as they can also react with the testes and ovaries.[186] 17 α-hydroxylase was the first autoantigen recognised in the sera of patients with APS1.[187] There is an increased likelihood of detecting this antibody in autoimmune Addison's disease associated with both concurrent and subsequent development of (POI).[53 185] Krohn et al showed that the presence of 17 α-hydroxylase autoantibodies is associated with autoimmune Addison's disease in children suffering from APS1[188] but subsequent work by Betterle et al indicates that 21-hydroxylase autoantibodies may be far more important as a marker of autoimmune Addison's disease in children.[183]

1.6.4 21-hydroxylase autoantibodies

The enzyme 21-hydroxylase is unique to the adrenal cortex and is located primarily within the zona glomerulosa.[189] The presence of 21-hydroxylase autoantibodies associated with clinical primary hypoadrenalism is diagnostic for autoimmune Addison's disease. However, in a proportion of patients, the autoantibody levels

decrease with disease duration, as only 50% are still positive 20 years after the onset.[190] The higher the titre, the greater the risk of the development of Addison's disease in the future.[191] In a multivariate analysis to determine at-risk individuals, high antibody titres versus medium-low titres were found with imminent evolution of autoimmune Addison's disease.[192] 21-hydroxylase autoantibodies are positive in 80% of APS1 and APS2.[190] Overwhelmingly, 21-hydroxylase autoantibodies are IgG1. A minority are IgG2 and IgG4, supporting a predominant Th1 immunological response.[145] While the predominant expression of IgG1 isotypes suggests a Th1 response, a small subset of patients with Addison's disease demonstrate a IgG 4 predominance suggesting a Th2 response.[193]

1.6.5 Antibodies to P-450 side chain cleavage enzyme (P-450 scc)

Antibodies to P-450 scc in isolated Addison's disease occur at considerably lower prevalence rates than 21-hydroxylase autoantibodies, but are similar to 17 α-hydroxylase autoantibodies.[194] The presence of P-450 scc autoantibodies is highly correlated with autoimmune Addison's disease and POI.[49] Of 263 Italian subjects with autoimmune Addison's disease, 35 (13%) had APS1, 107 (41%) had APS2, 13 (5%) had APS4 and 108 (41%) had isolated autoimmune Addison's disease.[49] The respective proportions of hypergonadotrophic hypogonadism and positive P-450 scc antibodies were 60% and 80% in APS1, 9% and 40% in APS2, 61% and 40% in APS4 and 0% and 20% in isolated autoimmune Addison's disease. In a study of APS1 by Soderbergh et al, P-450 scc occurred in 79% of patients with hypogonadism and an odds ratio of 6.8 for the association with Addison's disease.[195] Patients with POI and Addison's disease, in association with either APS1, APS2 or APS4 had positive P-450 scc antibodies in 93%, which was virtually identical to the prevalence of ACA and 21-hydroxylase autoantibodies in the same cohort.[185]

1.6.6 The occurrence of other autoantibodies

As Addison's disease frequently coexists with a cluster of other autoimmune conditions, it could be expected that multiple antibodies are discerned in serum; thyroglobulin and thyroperoxidase antibodies are the most frequently found. In an unselected group of 212 Polish patients with Addison's disease, (comprising 58 due to tuberculosis, 148 due to autoimmune and six with other adrenal disorders), 51 (24%) had positive microsomal antibodies and 67 (32%) had antithyroperoxidase antibodies.[196] In a cohort of 91 Dutch patients of whom 83 patients were considered to have autoimmune adrenalitis, 38 of these had at least one other autoimmune disorder. In addition to adrenal autoantibodies, microsomal antibodies occurred in 58%, antithyroglobulin in 23.4%, parietal cell antibodies in 19.8%, pancreatic islet-cell autoantibodies in 6.2% and ovarian antibodies in 3.7% of women[50] higher than the prevalence of other clinical autoimmune conditions. Autoimmune thyroid disease occurred in 26.5%, pernicious anaemia in 4.8%, T1DM in 1.2% and POI 7.3%.[51] In 1975, Irvine et al showed similar results to the Dutch study in 174 patients with autoimmune Addison's disease the prevalence of antithyroid autoantibodies was 59.7%, parietal cell antibodies was 28.7% and anti-ovarian antibodies was 22.9%.[197] In a review of APS in association with T1DM, which examined Belgian, Swedish and USA cohorts, Addison's disease occurred in 12-14%. Of the Addison's patients, 5% had positive tissue transglutaminase antibodies, 83-90% had 21-hydroxylase autoantibodies, and primary hypothyroidism was present in 14-21%, whereas anti-thyroperoxidase antibodies were present in 23-40%, Graves' disease was present in 10-20% and parietal cell antibodies were present in 6%. In the respective general populations, tissue transglutaminase occurred in 0.5-1%, 21-hydroxylase occurred in 0-0.6% and parietal cell antibodies 2.5-12%.[198] Individuals with autoimmune hepatitis and positive liver kidney microsomal 1 antibodies are prone to develop associated polyendocrinopathies, especially APS1. This is likely because of sequence homology between 21-hydroxylase and an enzyme P4502D6 expressed by hepatocytes, both of which can evoke an antibody response.[199]

1.6.7 Rationale to measure adrenal autoantibodies

Measurement of these autoantibodies helps to establish their prevalence in the healthy background population, the underlying aetiology of the adrenal failure and may identify patients at risk of Addison's disease, and so potentially avoid a life-threatening adrenal crisis.[49] The early detection of antibodies alerts physicians and so may help to avoid the full blown disease. Apart from parietal cell antibodies, the remaining autoantibodies occur at a sufficiently greater rate among Addison's patients than the general population, corroborating their usefulness as a screening tool.[200] The simultaneous presence of ACA and 21-hydroxylase autoantibodies in an individual with primary hypoadrenalism confirms autoimmune Addison's disease with 99% certainty.[55]

1.7 Clinical aspects of autoimmune adrenal gland failure

It is generally held that Addison's disease develops in association with other endocrinopathies in up to 50% of cases and 59% was found among 263 Italian autoimmune Addison's disease patients.[49] In order to diagnose APS1, two of the following criteria are needed: adrenocortical failure or serological evidence of adrenalitis, hypoparathyroidism and mucocutaneous candidiasis, while APS2 comprises adrenocortical failure with either autoimmune thyroid disease and/or T1DM. Three further polyglandular autoimmune syndromes have been described. In APS3 both autoimmune Addison's disease and hypoparathyroidism are absent; while in APS4 autoimmune Addison's disease occurs in the absence of both T1DM and autoimmune thyroid disease. The subdivisions of APS3 and APS4 are not universally used, and the majority distinguish APS as either APS1 or APS2, based on their respective ages of onset. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is a rare primary immune deficiency disease, manifesting in infancy and results in severe enteropathy, villous atrophy, dermatitis, T1DM and thyroiditis, and is caused by mutations in the Forkhead box P3 (FOXP3) gene, which is a master regulator in

the development and function of regulatory T-cells, inducing profound immune dysregulation.[201] Due to its rarity and its unlikely cause for Addison's disease in South Africa, it will not be discussed further.

1.7.1 Autoimmune polyglandular autoimmune syndromes

The clinical characteristics of each of the autoimmune polyglandular syndromes will be discussed below.

1.7.1.1 Autoimmune polyglandular syndrome (APS1)

Finland has the highest incidence of APS1 in the world and yet only 100 patients have been documented so far in a population of 5 million.[202] The major manifestations of APS1 are mucocutaneous candidiasis, which occurs within the first two years of life, hypoparathyroidism and adrenal insufficiency, chronologically usually following this order.[175 187] Minor endocrinological components of APS1 include primary hypogonadism and T1DM. Non-endocrinological manifestations include periodic malabsorption, gastric parietal cell atrophy, pernicious anaemia, hepatitis, alopecia, vitiligo, dental enamel hypoplasia, pancreatic exocrine dysfunction and keratopathy,[49 203] (Table 2).

Addison's disease occurs in more than 85% of patients and hypoparathyroidism usually presents before puberty.[49] Up to 64% of a Finnish cohort had these three major components of APS1 by the age of 30 years. Primary hypogonadism was the next most frequent manifestation, occurring in 60% of females and 14% of males. Up to 30% of patients had periodic malabsorption, gastric parietal cell atrophy, hepatitis, alopecia and vitiligo, in various combinations and at various time intervals. Of 263 Italian patients with Addison's disease occurring as part of APS (APS1, APS2, and APS4) or isolated Addison's disease, 100% had both 21-hydroxylase and ACA, 80% exhibited StCA, 80% had either 17 α-hydroxylase and/or P-450 scc autoantibodies.[49]

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1.7.1.2 Autoimmune polyglandular syndrome 2 (APS2)

The presence of autoimmune Addison's disease with either autoimmune thyroid disease or T1DM, constitutes APS2. Minor components include, inter alia vitiligo, chronic atrophic gastritis, hypergonadotropic hypogonadism, chronic hepatitis and Sjogren's syndrome.[49] (Table 2). The prevalence varies from 1.5-4.5 per 100 000 inhabitants, with mainly females being affected.[187] Thyroid autoimmune disease may be any of Hashimoto's thyroiditis, primary myxoedema, symptomless thyroiditis and Graves' disease. In a study by Neufeld et al, one of these thyroid manifestations occurred in 69% of patients with APS2, while T1DM occurred in 52% of the same cohort.[166] Among 107 Italian subjects with APS2, the prevalence of the minor components ranged from 1-11%, while T1DM occurred in 28%.[49] ACA and/or 21-hydroxylase autoantibodies were detectable in 100% of these cases, but StCA, 17 α-hydroxylase and/or P-450 scc autoantibodies were present in about 40% of the cases, in contrast to APS1 where the latter three autoantibodies were positive in about 80%.[49] The evolution of APS2 occurs with T1DM preceding Addison's disease, but autoimmune thyroid disease may occur prior, at the same time, or after the onset of Addison's disease.[204] There is merit in screening for at-risk individuals using autoantibodies because positive markers for

other endocrinopathies could facilitate prompt introduction of potentially lifesaving therapy. Although APS2 is inherited in an autosomal dominant pattern, multiple genotypes may predispose an individual to developing it, including HLA-B8, HLA DR3, HLA-DR 4, microsatellite polymorphism in the MHC class I chain-related (MIC-A) and CTLA-4, but results of studies are inconsistent.[49]

Table 2: The expected prevalences of autoimmune phenomena in autoimmune polyglandular syndromes (APS)[175]

	APS1	APS2
Type 1 DM	4-18%	41-52%
Autoimmune thyroid disease	8-40%	70%
Pernicious anaemia	12-15%	2-25%
Gonadal failure Males Females	 7-17% 30-60%	 5% 3.5-10%
Vitiligo	4-13%	4-5%
Alopecia	27%	2%
Autoimmune hepatitis	10-15%	Rare
Malabsorption	10-18%	Rare

Abbreviations:
APS1: autoimmune polyglandular syndrome type 1
APS2: autoimmune polyglandular syndrome type 2
T1DM: type1 diabetes mellitus
Adapted from Paediatric Endocrinology 2008 M Sperling, WB Sanders Philadelphia Autoimmune polyglandular syndromes Haller MJ, Winter W E, Schatz DA, page 770-787[175]

1.7.1.3 Incomplete autoimmune polyglandular syndrome 2 (absence of Addison's disease)

Incomplete APS2 is diagnosed in the absence of autoimmune Addison's disease, but it coexists with T1DM and autoimmune thyroid disease, or in a sibling that has Addison's disease and T1DM. The presence of T1DM, autoimmune thyroid

disease and positive ACA in an individual, should be considered as incomplete APS2.[49 166]

1.7.1.4 Autoimmune polyglandular syndrome 3 (APS3)

Autoimmune thyroid disease, in association with other autoimmune diseases and in the absence of both Addison's disease and hypoparathyroidism, is designated as APS3. Since it can occur with T1DM, autoimmune gastrointestinal involvement, autoimmune dermatological or an autoimmune connective tissue disorder, it may be sub-categorised by the presence of any of these aforementioned autoimmune conditions.[49]

1.7.1.5 Autoimmune polyglandular syndrome 4 (APS4) (autoimmune Addison's disease in the absence of both autoimmune thyroid disease and type 1 diabetes mellitus)

The combination of autoimmune Addison's disease and other autoimmune endocrinopathies, in the absence of both autoimmune thyroid disease and T1DM, constitutes APS4. The occurrence of ACA and 21-hydroxylase autoantibodies at the onset of Addison's disease is usually 100%. The autoimmune features include, inter alia; vitiligo, alopecia, chronic gastritis, pernicious anaemia, hypergonadotrophic hypogonadism and lymphocytic hypophysitis.[49 187]

1.7.2 Genetics of autoimmune polyglandular syndromes

The mode of inheritance differs between APS1 and APS2.

1.7.2.1 Genetic predeterminants of APS1

Specific components of the immune system, including: the thymus, lymph nodes, and peripheral blood cells including the CD14 positive monocytes, but not CD4 T-cells, express the AIRE gene. As the protein product of the AIRE gene is a transcription factor that functions to up-regulate certain organ-specific self antigens, it is an important mediator of central tolerance and is essential in the

negative selection of organ-specific thymocytes.[200] Transgenic mice with specific deletion of the insulin gene in medullary thymic epithelial cells, which express AIRE, develop autoimmune diabetes and insulitis, despite normal expression of insulin in the beta-cells. This latter experiment emphasises the role of the normal AIRE gene in maintaining central tolerance. APS1 is monogenic, resulting from mutations in the AIRE gene and is inherited in an autosomal recessive fashion. These mutations result in decreased expression of the transcription factor and diminished presentation of self antigens by the medullary thymic epithelial and dendritic cells to developing T-cells. This results in reduced central tolerance to a number of self antigens (Figure 6).[205] The most common is the R257X mutation responsible for 82% of Finnish and the majority of Italian APS1 alleles.[187] Other frequently encountered mutations that have been described include C311Y, P326Q and L397fsX478.[145] The L323fsX373 mutations of AIRE have been found to occur in Britain in association with APS1.[206] 21-hydroxylase, thyroid peroxidase and inter alia thyroglobulin are under the transcriptional control of AIRE.[174] High titres of antibodies to interferon-α and/or interferon-ω have been detected in subjects with APS1, but interestingly in none of their unaffected heterozygote relatives.[207]

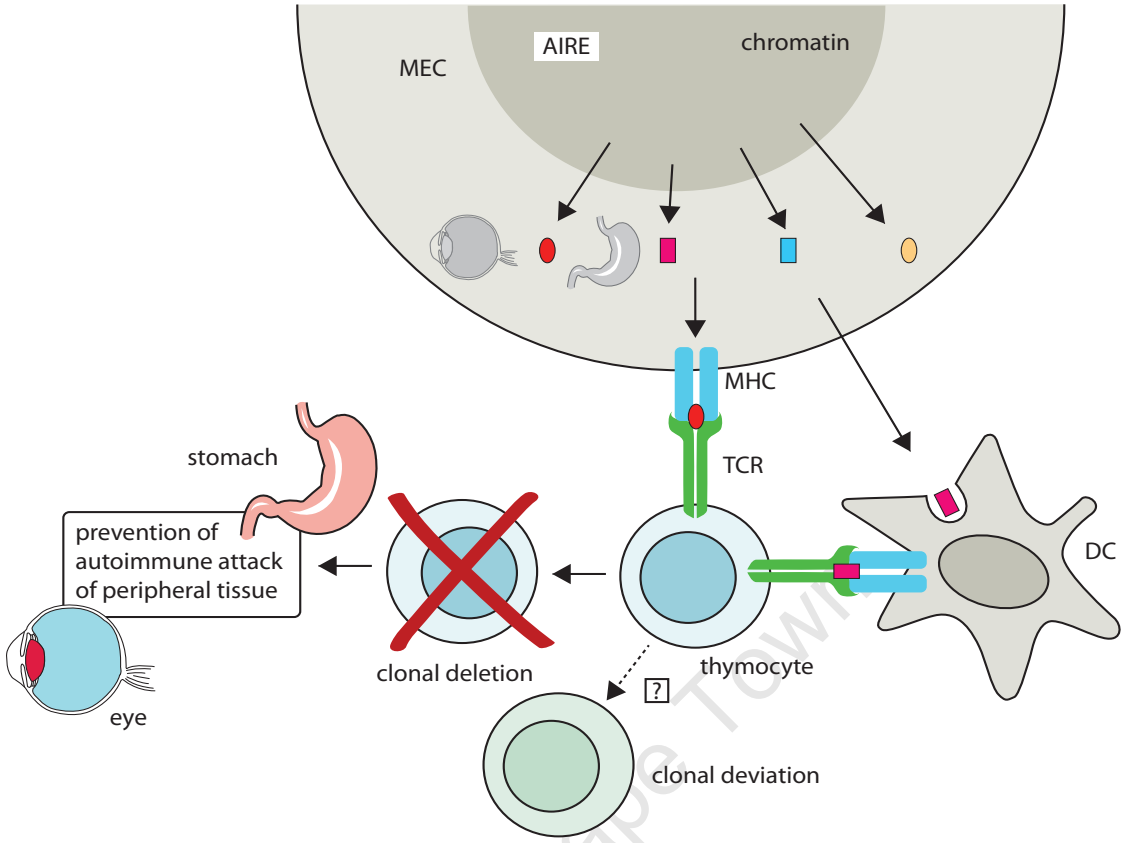

Figure 6: The mechanisms of the autoimmune regulator (AIRE) in preventing autoimmunity. Adapted from Mathis D, Benoist C.A decade of AIRE. Nat Rev Immunol. 2007;7(8): 645-50.[208] The function of the autoimmune regulator (AIRE) is to promote transcriptional activity of numerous chromosomal locations, with a view to stimulating expression of genes by medullary epithelial cells (MECs) in selected tissues. The antigens that are identical to peripheral self in MECs are presented to immature thymocytes either directly or by uptake of antigens released from MECs by thymic dendritic cells (DC). The differentiating T-cells, which recognise these antigens are removed by apoptotic clonal deletion. Some T-cells may survive by evolving regulatory cell function. Mutations in AIRE gene result in decreased expression of the transcription factor and less presentation of self antigens by the medullary thymic epithelial and dendritic cells to developing T-cells, which results in reduced central tolerance to a number of self antigens.

1.7.2.2 HLA (human leukocyte antigen)

This refers to a group of genes spanning 700 kilobases, located on chromosome 6p21.3 region-encoding cell surface and antigen presenting-proteins (Figure 7). Patients with certain HLA antigens have a higher propensity of developing autoimmune disorders such as T1DM, ankylosing spondylitis, systemic lupus

erythematosus (SLE) and myasthenia gravis. In the case of T1DM, 40-50% of the genetic risk may be explained by the DQ locus. An α- and β-chain of the MHC class II facilitate nine pocket-binding clefts; some are considered major and some minor. These create unique peptide binding patterns for various MHC molecules. The major MHC-binding pockets harbour polymorphic side-chain residues that determine which peptides will bind.[209]

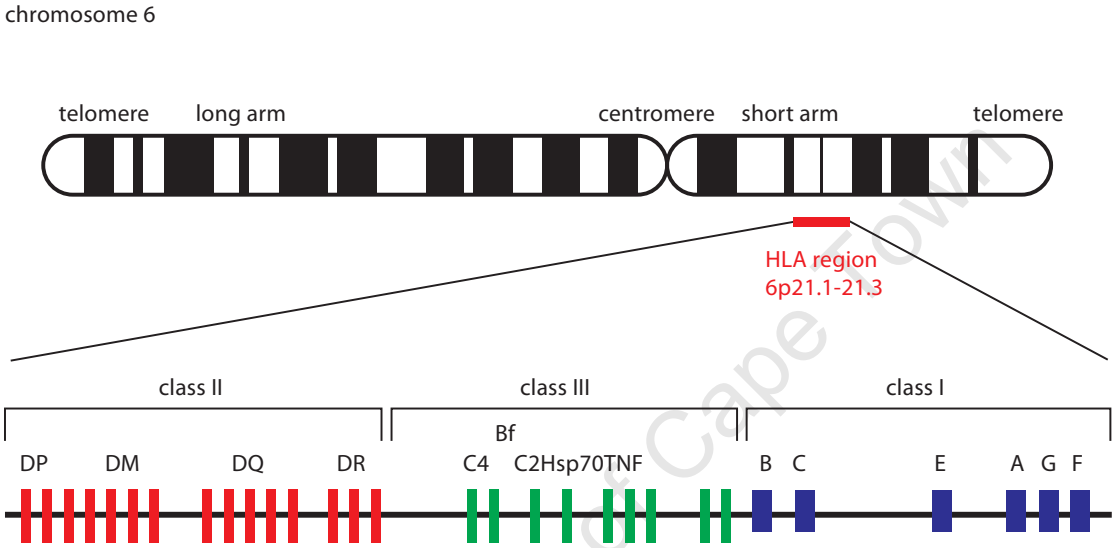

Figure 7: Gene map of the human leukocyte antigen (HLA) region. Adapted from Mehra NK, Kaur G. MHC based vaccination approaches: progress and perspectives. Expert Rev Mol Med. 2003 Feb 24;5(7):1-17. [210]The HLA region traverses 700 kilobases on the short arm of chromosome 6. HLA class I molecules restrict CD8 cytotoxic lymphocyte function and control immune responses against endogenous antigens and virally infected targets. HLA class II molecules are involved in the presentation of exogenous antigens to T-helper cells. The HLA class III region contains multiple genes encoding proteins that are not involved in cell-mediated immunity, but modulate immune responses using tumour necrosis factor (TNF), heat shock proteins and complement.

The α- and β-chains are encoded by HLA-DQA1 and HLA-DQB1, respectively and are both located on chromosome 6p21.3. Significant variation is likely to occur as normal individuals produce two α-and 2 β-chains. Thus, each person has four HLA-DQ isoforms. Each individual HLA-DQ isoform can present different antigens to the T-cells, which has the primary function of stimulating T-cells to maturity and in

turn signals B-cells to produce antibodies.[211] When there is a breach in tolerance, autoimmunity may result. HLA-DQ alleles influence susceptibility to T1DM and it is interesting that HLA DQB*0602 is protective.[212] Nevertheless, through linkage disequilibrium studies, it appears that particular DR/DQ allelic combinations are associated with autoimmune diseases.[213]

1.7.2.3 Genetic predeterminants of APS2

Autoimmune endocrinopathies arise because of immune-mediated destruction of either a single target endocrine gland or multiple endocrine glands. The development of APS requires a genetic susceptibility, an environmental trigger, active autoimmunity and metabolic derangements. Other genes and transcription factors, aside from HLA, are to a lesser degree responsible for the development of autoimmune endocrinopathies. APS2 appears to occur sporadically or in families of patients with APS2 and associates with HLA-DQB*0201 and DQB*0302. The HLA-DRB1*03-DQA1*0501-DQB1*0201 haplotype has such a strong association, that in all probability, could be considered a genetic marker for the APS2. HLA-DR3/DQ2 appears to be strongly associated with APS2 and even more consistently than HLA-B8 when it occurs as part of the HLA-B8-DR3 haplotype.[210] In a study of HLA genotypes DQ8/DQ2, consisting of DRB1*0404/DQ8 with DRB1*0301/DQ2 was present in 14 of 21 (67%) patients with Addison's disease and 8 of 12 (67%) with T1DM mellitus compared to 0.7% of the general population. This finding may be influenced by the common association of T1DM and adrenal autoimmunity, especially in the context of APS2. The genetic risk of Addison's disease associated with HLA is complex. In addition to DQB1 polymorphisms, HLA DRB1*0301, DQA1*0501, DRB1*0401, DQA1*0301 are positively associated while DRB1*0403 is negatively associated with Addison's disease.[214] Additionally, an association exists between the retrovirus-like long-terminal repeat (LTR) DQ-LTR13 and the development of autoimmune Addison's disease and T1DM, which is in linkage disequilibrium with HLA DQB1*0302 and HLA DRB1*0403.[215]

In addition to HLA genes, two non-HLA genes, such as the MHC class I chain-related A (MICA) 5.1 allele and the protein tyrosine phosphatase non-receptor type 22 (PTPN22) genes, may predispose a person to developing APS2. Specifically, the MICA 5.1 allele is associated with a genetic risk for Addison's disease, which is explained by the loss of the membrane-binding region of the protein and defective interaction with another integral membrane protein (NKG2D) critical for thymic maturation of T-cells. The combination of the microsatellite allele 5.1 located in class I of the HLA antigen and HLA DR3/DQ2 appear to be quite important in the genetic risk of autoimmune Addison's disease is identified in a group of Italian patients.[216] The PTPN22 gene appears to regulate T-cell receptor signalling and a polymorphism within this gene, resulting in a gain of function, yields decreased T-cell receptor signalling and increased risk of autoimmune disorders.[174] SNP's of the PTPN22 gene have demonstrated conflicting associations with autoimmune Addison's disease and in a large cohort of UK entirely subjects, a specific SNP C185T was found to occur more commonly in autoimmune Addison's disease versus healthy control subjects (12.2% vs. 7.8%; $p < 0.008$). A meta-analysis of three further patient cohorts indicated an OR of 1.44, CI (1.21-1.72) for autoimmune Addison's disease.[217] Class II expression on APCs including B-cells, monocytes, dendritic cells and activated T-cells, is under the control of the class II transactivator. Two European studies have demonstrated the genetic association between polymorphisms of the class II transactivator and the development of autoimmune Addison's disease, independent of HLA gene markers, by conferring variations in the level or tissue selectivity of MHC class II expression.[190] Class II expression is under control of the MHC class II transactivator encoded by the MHC2TA gene. An A-G SNP was suggested to confer susceptibility to autoimmune diseases. Both heterozygous and homozygous polymorphisms of the rs3087456 SNP have been associated with a genetic risk of autoimmune Addison's disease.[218] In addition, a C-type lectin domain family 16, member A (CLEC 16A) was found to be independently associated with Addison's disease, after having been found in a genome wide association study of other autoimmune disorders.[219] Others

have reported an association between vitamin D receptor and the cytochrome P450 family, subfamily B, polypeptide 1 (CYP27B1) gene, as well as the NACHT leucine-rich-repeat protein 1 (NALP1) gene in the predisposition to APS2.[145] As shown HLA alleles are not the only genetic influence in the development of APS2 and genome-wide scans may be necessary in the future to determine the likely additional genetic risk of developing this condition.

Indeed several negatively associated genetic markers have been identified. HLA DRB1*0403 is a strongly protective marker for both T1DM and Addison's disease in Italy.[220] Several HLA class II including DRB1*01-DQA1*01-DQB1*0501 and DRB1*13-DQA1*0103-DQB1*0603 were negatively associated with Addison's disease.[221]

1.7.3 Genetic predeterminants of type 1 diabetes mellitus, as an example

The major genetic determinants of T1DM are located within the MHC on chromosome 6p21.3. Specifically, class II genes encoding HLA-DR and HLA-DQ contribute the most towards the susceptibility of developing T1DM through a simple recessive inheritance pattern.[222 223] More than 90% of Caucasian patients with type 1A diabetes have either DR3,DQ2 (DQ2 = DQA1*0501, DQB1*0201) or DR4,DQ8 (DQ8 = DQA1*0301, DQB1*0302) haplotypes.[223] Between 30% and 50% of patients with type 1A diabetes are heterozygotes for DR3 DQ2 /DR4 DQ8 compared with 2.4% of the general population.[224] In Denver, Colorado only 2% of newborns are HLA DR3/DR4 heterozygotes, whereas 30% of children with type I A diabetes have this genotype.[225] By contrast, specific DR and DQ alleles in an autosomal dominant pattern can confer protection against evolution of type 1A diabetes. The most common protective alleles are DQA1*0102 and DQB1*0602. This was determined by the finding that approximately 20% of control populations have these alleles, compared with fewer than 1% of children with type 1A diabetes mellitus.[226 227]

1.8 Screening for primary adrenal failure

The short synthetic adrenocorticotrophin (ACTH) is regarded as the most reliable diagnostic test for chronic adrenal hypofunction. Indeed its excellent sensitivity and specificity has confirmed its use in screening for hypoadrenalism in persons at risk for developing Addison's disease.[228]

The adrenal cortex, responsible for the release of cortisol and androgens, is under the negative feedback control of ACTH, while the release of aldosterone is controlled by the renin-angiotensin II system. Progressive functional deterioration, as in autoimmune destruction of the adrenal cortex, results in diminished function adrenal reserve as shown by reduced plasma concentration of cortisol and aldosterone in association with an increased ACTH and renin release.[49] The inability of the adrenal cortex to respond to synthetic ACTH may be due in part to the cortex being maximally stimulated by endogenous ACTH. Synthetic ACTH injected intravenously or intramuscularly, irrespective of whether the 1 μg or 250 μg doses are administered, is vastly supra-physiological. Subjects who have either basal or stimulated cortisol levels of >500 nmol/L do not have overt Addison's disease.[2 229] The short synthetic ACTH stimulation test can be used to diagnose primary and secondary hypoadrenalism. A meta-analysis showed that the low-dose (1 μg) and the standard dose (250 μg) ACTH stimulation test performed similarly, but the receiver operating curves using the 1 μg performed slightly superiorly in ruling out HPA insufficiency. However, the differences were clinically unimportant.[230]

Betterle et al, proposed that subclinical autoimmune adrenalitis may evolve through 3 defined stages: stage 1 where both ACTH and basal cortisol levels are normal, with concomitant elevated plasma renin activity and reduced or normal aldosterone levels; stage 2, when ACTH levels are normal, but the peak stimulated cortisol level is reduced, and stage 3 when the ACTH level is elevated through compensation, but the basal cortisol is reduced. Should stress, surgery,

pregnancy, infection or trauma coexist in stage 3 of subclinical disease, overt clinical hypoadrenalism may manifest. Adrenal cortical mass declines throughout these predetermined subclinical stages.[49] Subclinical hypoadrenalism should be considered when the ratio of ACTH to cortisol is increased. This is evidence in early autoimmune primary adrenal insufficiency, where patients exhibit reduced sensitivity to low-dose ACTH stimulation.[231]

The presence of either circulating ACA or 21-hydroxylase antibodies however does not invariably indicate that adrenalitis or incipient hypoadrenalism will occur. For example, a baby born to a mother who had ACA and 21-hydroxylase autoantibodies, Addison's disease, and co-existing hypothyroidism (APS2), did not develop either clinical or subclinical hypoadrenalism until 34 months of age, suggesting that an additional factor other than positive adrenal autoantibodies is required to induce Addison's disease.[232] In Padua, Italy, the cumulative risk for developing autoimmune Addison's disease was 48.5% among ACA positive subjects,[191] identifying a sub-group in whom screening needs to be performed.

1.8.1 Hypothesis of adrenal failure and genetic predisposition in South Africa

In a South African retrospective analysis of 50 patients who presented to a teaching hospital with acute Addison's disease, autoimmunity as a cause was uncommon.[233] However, it is hypothesised that autoimmunity in a large South African cohort with Addison's disease may be far more common if defined by the presence of circulating ACA and 21-hydroxylase autoantibodies. Moreover, certain HLADQB1 genotypes may predispose people to autoimmunity Addison's disease in South Africa.

1.9 Lipids, lipoproteins and markers of cardiovascular disease in primary hypoadrenalism

1.9.1 Introduction

It has been contended that survival of patients with Addison's disease on replacement therapy has been comparable to that of the background population. The overall mortality rate was the same in a Norwegian study as the background population, a sub-group of Addison's patients younger than 40 years of age, particularly in males, was at risk of premature death due to infections, sudden death and acute adrenal failure.[234] On the other hand, a Swedish publication reviewing death registers documented a relative risk of death of 2.19 for all causes, indicating a two-fold risk for premature death, in appropriately treated Addison's disease patients. The risk ratio for death from cardiovascular disease (CVD), the leading cause of death, was 1.97 for men and 2.31 for women, with ischaemic heart disease, followed by cerebro-vascular disease.[235] Moreover, a similarly conducted Swedish study of primary autoimmune adrenal failure corroborated a more than double mortality rate secondary to CVD.[236] The Norwegian study was based on a population registry, on the other hand both Swedish studies were based on registries of hospitalised patients and the accuracy of either diagnosis or aetiological classification were not verified. The reasons for this accelerated mortality associated with CVD were not investigated. It is possible that Addison's disease per se may have contributed to the excess mortality, although exposure to supra-physiological GCs with resultant hypertension, T2DM and obesity with centralisation of body fat and abnormal plasma lipids may be contributors.[237] The role of these factors has not been explored in Addison's disease. However, GC replacement dose reduction from 30 mg to 15 mg in hypopituitary patients has not invariably resulted in an improved CV risk factor profile.[238] GC replacement, irrespective of the dose, may fail to mimic endogenous cortisol levels with supra-physiological peak levels in the morning and evening soon after its administration,

yielding yet another explanation for accelerated CVD. [239]

In contrast to hypopituitarism, there is a paucity of studies appraising CVD risk in primary hypoadrenalism, apart from the study by Giordano et al, which demonstrated higher total cholesterol (TC) and TG levels, than controls in a study of 38 Addison's patients.[240] In another study, DHEA administered to Addison's patients resulted in no improvement in lipids and lipoproteins, although these were not abnormal at base-line.[241] A meta-analysis demonstrated a higher mortality rate, especially among women with hypopituitarism, due to inter alia CVD.[242] GH deficiency is the most likely culprit for accelerated CVD in hypopituitarism judging by the large number of CVD risk factors that normalise on GH replacement, independent of the dose of GC replacement.[243]

The traditional risk factors for CVD and cerebral vascular disease include hypertension, tobacco exposure, hypercholesterolaemia and diabetes mellitus.[244] More recently, inflammation has been implicated in the pathogenesis of atherosclerosis. Several serum markers of inflammation have been proposed to indicate or play a role in the pathogenesis of atherosclerosis.[245]

A brief overview of lipid and lipoprotein metabolism and the effects of GCs will follow. Lipids and lipoproteins are dynamic in nature and are subject to multiple fluxes, with influences at multiple points in their metabolism. Not only genetic variations, but also environmental factors influence the steady state and response to a given stress. It is therefore not always possible to predict confidently a net effect of a single stimulus.

1.9.2 Lipoprotein metabolism

1.9.2.1 Introduction to lipid metabolism

Lipoproteins transport lipids from their sites of production and are modulated by various enzymes, apoproteins and transfer factors. There are four major

pathways involved in lipoprotein metabolism; (i) the post-prandial pathway for chylomicrons, which transport dietary lipids, (ii) the constitutive pathway involving very low density lipoprotein (VLDL) for TG transport, (iii) the provision of LDL cholesterol, and (iv) the reverse cholesterol transport system, which involves HDL.[246] These pathways and the reported effects of GCs are shown in Figure 8.

1.9.2.2 Post-prandial pathway of chylomicrons for dietary lipid transport

Chylomicrons consist of 85-90% TG and are produced in the epithelial cells of the small intestine. Their apoproteins (apo) are mainly B48, apoAI and apoAIV. Hydrolysis of TG in chylomicrons occurs at the vascular endothelium where lipoprotein lipase is anchored on cells by heparan sulphate proteoglycans. This results in remnants, which are now proportionately richer in cholesterol esters. These chylomicron remnants by virtue of their large size, have low atherogenic potential, though they may still penetrate the arterial wall. The chylomicron remnants, which possess apoE, are cleared rapidly by the liver from plasma, by their ability to interact with remnant receptors in the liver. [247] [248]

1.9.2.3 The constitutive pathway of VLDL

The liver secretes VLDL, which comprises 55% TG, 20% cholesterol esters, 15% phospholipids and 10-15% protein. Its production and secretion is enhanced by uptake and circulation of free fatty acids (FFA), whether generated by intravascular lipolysis or by adipocytes. VLDL is assembled on apoB100.[249] [250] The cholesterol TG phospholipid components of VLDL may be produced de novo by the liver or could be re-used from uptake of lipoproteins. The TG content of VLDL is also hydrolysed by lipoprotein lipase. TG in VLDL remnants is hydrolysed by hepatic lipase, forming progressively smaller particles and becoming richer in cholesterol, first as intermediate dense lipoproteins (IDL) and ultimately as LDL.[251]

1.9.2.4 The LDL pathway

LDL contains most of the cholesterol in the plasma. The components of LDL are

35% cholesteryl ester, 10% free cholesterol, 10% TG and 20% phospholipids. The remaining 25% is protein; almost completely apoB100. The vast majority of LDL is taken up in hepatocytes, through the LDL receptors. Plasma LDL concentration may be raised by increased production of VLDL or through decreased clearance by down-regulation of LDL receptors. ApoE-containing lipoproteins (remnants) compete with LDL for uptake at LDL receptors on the hepatocytes.[252]

1.9.2.5 The reverse cholesterol transport system involving HDL

High density lipoprotein (HDL) is the smallest of the lipoproteins. About half comprises lipids, 25% phospholipid and 15% cholesteryl ester. Free cholesterol and TG both constitute 5%. The remaining half is made up of proteins apoAI and apoAII are the major apolipoproteins of HDL. The liver secretes apoAI-containing phospholipid discs, as nascent or immature HDL particles. The intestine is also able to synthesise small particles that contain apoAI. Additionally, HDL may result from the metabolism of chylomicrons and VLDL through lipolysis.[253] The enzyme lecithin-cholesterol-acyl-transferase (LCAT) esterifies cholesterol to a long chain fatty acid from phospholipids in the shell around the particles. Migration of cholesteryl esters to the core leads to a spherical particle that is viewed as a more mature form (HDL3). With progressive increase in size, LCAT transforms HDL3 into the larger and less dense HDL2. Cholesteryl ester transfer protein (CETP) transfers cholesteryl ester from HDL2 to VLDL and IDL, permitting the delivery of cholesterol to the liver by this route. In exchange, HDL receives TG.[253] Hepatic lipase hydrolyses the TG and thus regenerates the smaller HDL3 particle for more cycles of cholesterol esterification and enlargement, followed by lipolysis. Exchange of TG into LDL could result in a similar modulation of LDL to a smaller particle. The esterification of surface-free cholesterol in HDL creates space for more free cholesterol to be accepted from cells or other lipoproteins. HDL can also deliver cholesterol directly to the liver, leading to the secretion of cholesterol and/or bile acids, some of which would be excreted from the body.[254]

1.10 Lipid changes with corticosteroid use

1.10.1 Lipid pathways affected by glucocorticoids

Despite the frequency with which pharmacological doses of GCs are used, there are surprisingly few publications on their effects on lipid metabolism. The effects gleaned from the literature are indicated in Figure 8.

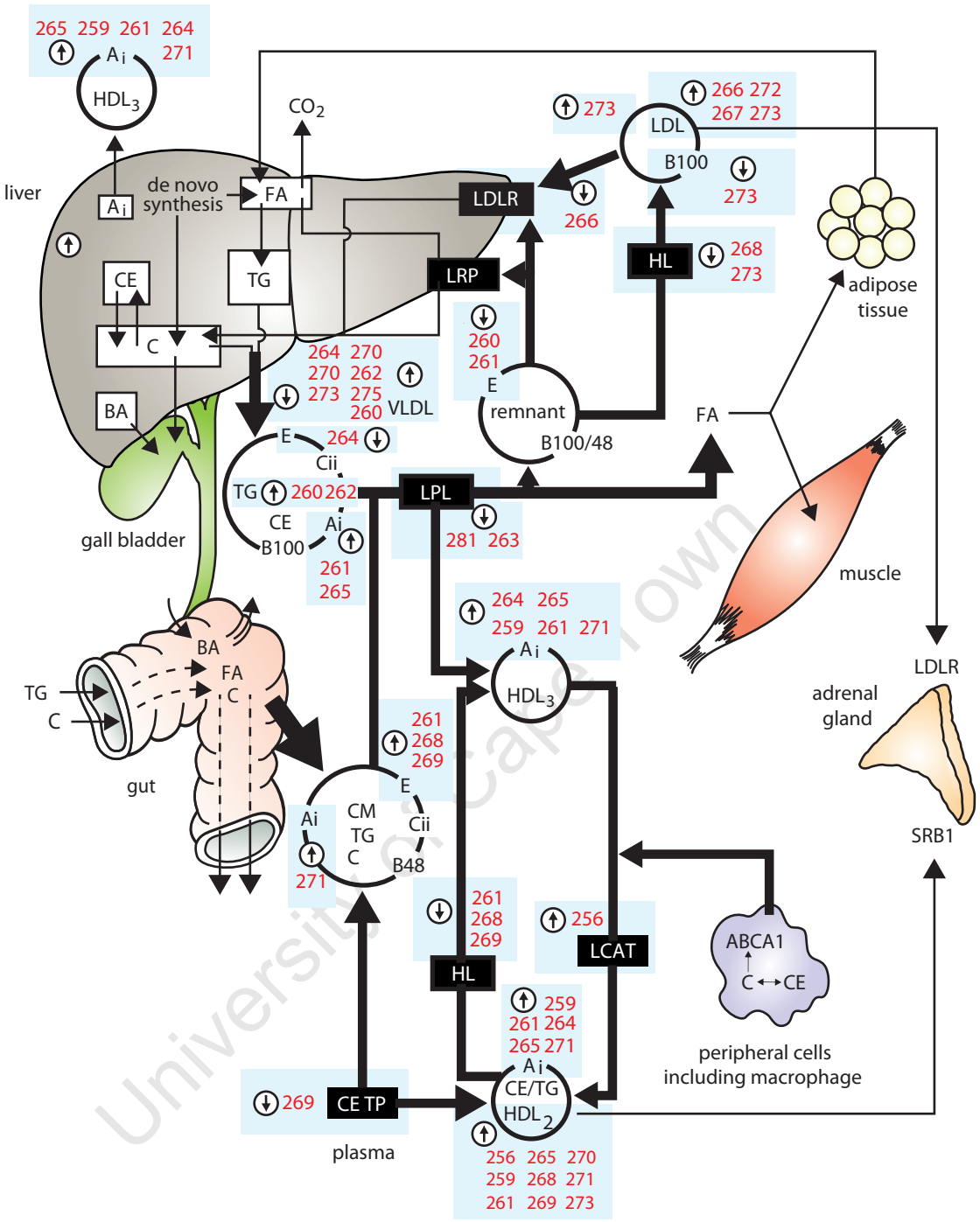

Figure 8: A schematic view of lipoprotein metabolism and where points of interaction with glucocorticoids have been reported in the literature. Adapted from Marais AD Lipids, lipoprotein metabolism and their derangements. South African Heart Journal September 2005, volume 2 number 3 page 8-18. [246]

Abbreviations:

Ai: apolipoprotein–AI

HDL_3: high density lipoprotein 3

HDL_2: high density lipoprotein 2

CE: cholesterol ester

C: cholesterol
BA: bile acid
TG: triglyceride
NEFA: non-esterified fatty acids
apoE: apolipoprotein E
B100: apolipoprotein B100
Cii: apolipoprotein CII
LDL: low density lipoprotein
LDLR: low density lipoprotein receptor
LRP: low density lipoprotein receptor protein
CM: chylomicron
LPL: lipoprotein lipase
CETP: cholesterol ester transfer protein
HL: hepatic lipase
LCAT: lecithin cholesterol acyltransferase
SRB1: scavenger receptor B1
ABCA1: adenosine binding cassette transporter A1
VLDL: very low density lipoprotein
FA: fatty acids

These effects are also summarised in Table 3. Reference numbers provided in the table are also given in red print and appear in the reference list.

Dyslipidaemia and hypertension are the most significant CV adverse effects resulting from pharmacological doses of GCs.[255] The precise mechanisms by which GCs alter lipid metabolism are not clear. There are conflicting data about GC action on lipids and lipoprotein metabolism. Changes in lipid profiles in humans have been documented on varying doses of prednisone.[256-259] The major changes that have been reported are: elevated VLDL and TG either increased or decreased high-density lipoprotein cholesterol (HDLC) and increased low-density lipoprotein cholesterol (LDLC).

1.10.1.1 Animal studies concerning lipid changes and steroid use

A single dose of hydrocortisone administered to rabbits with established

atherosclerosis, raised plasma TG significantly, but not the TC concentration[260] suggesting an increased production of TG-rich VLDL or possibly decreased metabolism. GCs reduce hepatic lipase activity and thereby reduce the metabolism of HDL2 to HDL3, resulting in elevated HDLC. Rats exposed to dexamethasone and triamcinolone have shown increases in TC and TG, but these findings were not duplicated with hydrocortisone.[261] When hydrocortisone was administered to rats at a fixed dose of 100 μg/g of body weight, there was a reduction in total plasma cholesterol. Plasma apoAI was increased and apoE was decreased, but a differential effect was noted on plasma apo levels using hydrocortisone, triamcinolone and dexamethasone. The greatest increases were observed in apoAI, using triamcinolone and dexamethasone. The latter also yielded the largest increase in apoAIV, and triamcinolone caused the greatest increase in apoE levels. In contrast, the greatest reduction in plasma apoE levels was found in rats receiving hydrocortisone.[261] Decreased remnant clearance would raise plasma TG, and could provide particles similar to LDL and promote atherosclerosis. Reaven et al, demonstrated that methyl-prednisone administered to normal rats for 8 days resulted in increased levels of TG and almost double the amount of the plasma cholesterol.[262] A reduction in lipoprotein lipase activity from adipocytes was reported to occur in response to GCs, resulting in an increase in TG and a decrease in HDLC.[263] Plasma apoE levels decreased on exposure to hydrocortisone, either as a consequence of decreased hepatic secretion or increased catabolism of apoE-containing lipoproteins. Additionally, lower production of apoE by extra-hepatic tissues has been suggested.[264] Various organs, including the brain, spleen and kidney produce apoE, where its major function is thought to be the redistribution of cholesterol from cells with excess to those that require cholesterol.[264] Plasma apoAI has been found to increase with most GCs, but the effect is considerably greater on exposure to triamcinolone and dexamethasone, resulting in increased HDLC.[261 264] An increase in hepatic apoAI mRNA has also been documented in cultured rat hepatocytes exposed to GCs.[265] Transient down-regulation of LDL receptors has been documented

in rats, following administration of methyl-prednisolone to account for elevated levels of LDL and TC.[266]

1.10.1.2 Human studies concerning lipid changes and steroid use

A positive correlation has been shown between plasma LDLC and endogenous plasma cortisol in healthy men aged between 52 years and 67 years of age.[267] Unless other changes occur in Addison's disease, it can be speculated that GC deficiency will lower LDLC. GCs alter plasma lipids within 14 days.[259] A study examining the acute effects of dexamethasone (3 mg), administered twice daily (equivalent to an acute stress), to young men aged between 19 years and 39 years of age on CVD bio-markers, reported lower hs-CRP levels, increased HDLC and no alteration in LDLC, FFA and TG.[268] In the post-cardiac transplantation setting, GCs have also been found to reduce hepatic lipase activity and impair CETP, and thereby could raise HDLC and reverse cholesterol transport.[269] Another study reported that GCs reduced the level of hepatic lipase activity.[268] Beentjes et al found that GC prescription in hypopituitary patients resulted in low VLDL, reduced LDLC, and reduced LCAT and CETP.[270] Serum lipid levels were evaluated among US adults, using GCs in the third National Health and Nutrition Examination Survey (NHANES), which demonstrated that GC use was associated with higher HDL levels and lower ratios of total cholesterol-to-HDL.[271] Both GC use and endogenous hypercortisolism such as Cushing's disease, have resulted in elevated TC and LDLC. [272]

Based on animal and human studies, exposure to GCs may produce either increased or decreased HDLC and could disrupt reverse cholesterol transport flux. Some studies corroborate up-regulated hepatic LDL receptor activity, which explains a decrease in LDLC. [273] [274] While GCs are known to have pleiotropic actions on multiple physiological and pathological processes, it would appear that there could be varied responses to the lipid and lipoprotein homeostasis and that some of these changes could be atherogenic. These findings are summarised

in Table 3, as can be best deduced from the various strategies used to identify lipoproteins and mechanisms of change.

Table 3: Changes in lipid and lipoprotein metabolism attributable to glucocorticoid treatment

Lipid parameter	Effect (1) Increase	Effect (2) No Change	Effect (3) Decrease
TC (composite of all lipoproteins)	262 266 272 275	263	270 in hypopituitary individuals
VLDL (Reflects most of fasting plasma TG)	259 260 262 275 260rabbits; increased TG by 80%; increased VLDLC 262rodent increased VLDL size 263decreased lipoprotein lipase activity responsible for increased TG 275Remarkably supra-physiological dose used	261 268	270 273
LDLC (Bulk of plasma cholesterol in humans) LDL particle size	266 267 275 266reduction in LDL receptor mRNA 267human plasma cortisol proportional to LDLC 272human study; Cushing's disease Increased small dense LDL	275-278 273 dexamethasone	273 Corticotropin decreased LDLC and apoB Decreased small dense LDL273

Lipid parameter	Effect (1) Increase	Effect (2) No Change	Effect (3) Decrease
HDLC (contains apo AI and substrate for LCAT & CETP)	[256 259 261 263-265 271 272] [256]low-dose glucocorticoids in women with rheumatoid arthritis apoAI was unchanged, but HDLC increased by 15% [260]apoAI increased by 18% and HDLC increased by 28% following prednisone after 2 weeks [261]apoAI increased with hydrocortisone, triamcinolone and dexamethasone variably, but only dexamethasone increased apoAIV in rats [265]apoAI increased after exposure to dexamethasone [268]increase of HDLC by 10% [269]increased phospholipids, unesterified cholesterol and apoE, reduced CETP& hepatic lipase, LCAT unchanged [271]apoAI significantly higher among glucocorticoid users; atheroprotective ratios in elderly [273]increased after corticotropin and dexamethasone in healthy humans [270]In human hypopituitary patients		[272] promotes atherogenic ratio

References for studies: human[256 259 267-272], rodent[261-263 265,] rabbit[260]
References in superscript

1.10.2 Medical conditions due to steroid excess

Dyslipidaemia, hyperglycaemia and hypertension are the most significant CV adverse effects that result from pharmacological doses of GCs.[279] Cushing's syndrome provides a pathological state in which to evaluate lipid abnormalities in relation to excess steroids. The earliest work from 1983 reported markedly elevated mean levels of TC, TG, LDLC and HDLC, prior to treatment in 11 women with Cushing's syndrome, and all parameters improved after successful

removal of the autonomous cortisol secreting tumour.[280] It was suggested that hypercortisolism stimulates the production of hepatic VLDL particles.[281] Lipid abnormalities, that are: elevated TC, LDLC and TG, but a low HDLC, have also been found in sub-clinical Cushing's syndrome.[281 282]

1.10.3 Medical conditions requiring glucocorticoids for therapy

Lower than normal HDLC levels are seen in untreated rheumatoid arthritis patients and the inflammation per se, may induce these abnormalities,[283] as part of the short-term acute phase encountered in the majority of subjects. Rheumatoid arthritis may represent a unique clinical scenario where some of the adverse lipid profile may be dampened by GCs.[256] Rheumatoid arthritis represents an independent risk factor for CVD. In a recent meta-analysis of 24 studies involving 111 758 patients there was a significant increase of CVD. The mortality from CVD and cerebrovascular disease in this meta-analysis increased by 59% and 50% respectively, compared with the general population.[284] Reported changes in the lipid profile in rheumatoid arthritis include increased or decreased cholesterol levels, but it should be borne in mind that the populations differed in terms of disease activity, and not all patients were treated with GCs.[285-288] Patients with rheumatoid arthritis were reported frequently to have high TC and LDLC, and decreased HDLC.[256 289] On the other hand, a reduction in plasma apoB100 concentration has been identified among patients with active rheumatoid arthritis.[290] This may well reflect an acute phase response.

A rise in TG suggests VLDL overproduction, with consequent exchange of TG into HDL and remodelling, explaining small HDL particle size and low HDLC. Similarly, LDL would remodel into smaller particle size. Frequently low doses of GCs (5 mg prednisone) are administered over a long period of time to rheumatoid arthritis sufferers. One study reported increased HDLC in response to GCs, without affecting the TC, TG and IDLC. Higher HDLC was attributed to a rise in HDL2 sub-fraction.[256] Patients with SLE are at increased risk of developing CVD.

Accelerated atherosclerosis has been attributed to SLE itself and/or to the GC therapy. Recent exposure to GCs, along with an increased activity index in SLE, conferred the greatest risk for atherosclerosis.[291]

Hypopituitarism differs from Addison's disease, in that growth hormone deficiency, exposure to radiation and multiple endocrine deficiencies may coexist. Some reports show that hypopituitary patients on conventional replacement therapy (hydrocortisone, thyroxine and sex steroids) are subject to increased morbidity and mortality as a consequence of accelerated atherosclerosis[276 292] but the exact pathophysiology is unknown. Hypopituitary patients who were thought to be optimally replaced by their individual clinicians, {mean total daily hydrocortisone dose of 27 mg (with a range from 12.5 mg to 35 mg)}[293] had adverse lipid profiles of increased TG, increased TC and increased LDLC, compared to the controls.[276] The authors speculated that the hydrocortisone replacement may have contributed to these abnormal lipid profiles. A cyclic variation in TG was identified among women. It was lowest between 01h00 and 07h00 and highest between 07h30 and 20h30. Additionally, minor elevations in TC were observed postprandially.[276] A reduction in hydrocortisone doses from 20-30 mg per day to 10-15 mg per day resulted in an average loss of 7.1 kg of body fat in 11 patients not receiving growth hormone. The TC and TG decreased significantly, indicating the beneficial effect of reducing GC replacement doses on lipid profiles, independently of growth hormone supplementation.[275] The coronary risk in growth hormone-deficient hypopituitary patients has been attributed to abnormal lipid profiles.[277] Males had raised BMI, TC, LDLC and TG, compared to the controls. Among females, the BMI and TG were significantly raised, while the HDLC was lower compared to the controls in hypopituitary patients receiving growth hormone.[278 294] Various doses of hydrocortisone supplementation in growth hormone-replaced patients were studied. Doses of less than 20 mg of hydrocortisone per day had the least (unfavourable) metabolic consequences.[294]

1.11 The relationship between diabetes mellitus, dysglycaemia and abnormal lipid profiles

Either T1DM or T2DM can coexist with Addison's disease as part of APS and the metabolic syndromes respectively. Abnormal lipid metabolism, usually manifested as hypertriglyceridaemia, is frequently encountered in diabetes and is a function of glucose control.[295] The risk of CVD events in diabetics is the lowest when the glucose level is between 4.0 mmol/L and 4.9 mmol/L.[296] The EDIC study demonstrated the benefits of good glycaemic control and reduction of CVD events, even though the glycosylated haemoglobin A1C (HbA1C) did not differ between the group with initial optimal glycaemic control compared to the less intensively treated group.[297]

Hypertriglyceridaemia, the commonest lipid disorder among diabetics, is established as an independent risk factor for CVD.[298 299] In the UK Prospective Diabetes Study (UKPDS), the clinical risk factors in newly diagnosed T2DM at diagnosis were compared with age-matched normal subjects who did not have any first-degree relative with diabetes. The diabetic patients were more overweight and the TG levels were higher.[300] The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) investigators examined the effect of Fenofibrate on reducing the CVD events in diabetes. The combination of TG >2.3 mmol/L and low HDLC levels, conferred the greatest risk of CVD at 17.8% at 5 years.[301] Patients with impaired glucose tolerance were also found to have increased TG, as shown in the Diabetes Epidemiology Collaborative Analysis of Diagnostic Study in Europe (DECODE), which explored the relationships between lipid levels and glucose status in European subjects without a prior history of diabetes. Age is an important pre-determinant of fasting hypertriglyceridaemia.[302] It has been shown that TG >1.7 mmol/L occurs in up to 30% of individuals that are 20 years of age and continues to rise to about 43% for those that are 50 years of age and over, irrespective of diabetic status.[303] HDLC is frequently low in T2DM, which compromises its athero-protective action.[303] Women with impaired

fasting glucose had lower HDLC concentrations and higher TC/HDLC ratios than compared to the controls.[304]

1.12 Biochemical markers of cardiovascular inflammation

A number of inflammatory serum markers predict CVD. Of more recent interest are: highly sensitive C-reactive protein (hs-CRP), serum amyloid A (SAA) and lipoprotein-associated phospholipase A2. Numerous cross-sectional and longitudinal studies have identified CRP as a predictor for CVD. hs-CRP is associated with a 2-3 fold increase in the prevalence of myocardial infarction, stroke and peripheral vascular disease[305] and predicts CVD events in patients with or without antecedent events.[306] Its function as a reliable predictor for CVD events has been verified through studies such as the West of Scotland Coronary Atherosclerosis Prevention Study (WOSCOPS)[307] and the Framingham heart study.[308] Patients with the metabolic syndrome have higher levels of hs-CRP, than those without the metabolic syndrome.[309] The relationship between the individual components of the metabolic syndrome and hs-CRP was investigated. In a cross-sectional multicentre study, a population-based survey was conducted to determine the prevalence of CV risk factors among 1344 subjects who fulfilled the Adult Treatment Panel (ATP) III diagnostic criteria for the metabolic syndrome. The prevalence of the metabolic syndrome was 30.9% (95% confidence interval 28.4%-33.3%). Each individual component, except for HDLC, was associated with an elevated hs-CRP, with waist circumference exhibiting the strongest association with hs-CRP. Moreover, a gradual increase in the log transformed levels of hs-CRP was observed, with an increasing number of components of the metabolic syndrome. [310]

Serum amyloid A (SAA) is linked to atherosclerosis risk. SAA leads to expression of proteinases, which degrade extracellular matrices.[311] The concentration of SAA in the blood increases dramatically during acute inflammation. SAA becomes

enriched in HDLC at the expense of apoAI. Phospholipase A2 enzymes hydrolyse phospholipids, generating lysophospholipids and FFA.[312] Lipoprotein associated phospholipase A2 is preferentially found on small LDL.[313]

Non-esterified fatty acids (NEFA) are released from adipose tissue by the action of hormone sensitive lipase. This enzyme is not suppressed in diabetics and NEFA concentration may parallel that of deteriorating glycaemic control.[314] The association of hormone-sensitive lipase and NEFA is strong.[315] An association also exists between high NEFA in offspring and increased risk of CVD in their parents. NEFA may have a direct pro-arrhythmogenic effect and predispose individuals to sudden cardiac death presumably arrhythmogenic by shortening the action potential duration, through activating the A.T.P.-sensitive potassium channels.[314] As circulating NEFA are to a large extent influenced by the adrenergic tone, it is possible that the adrenergic stimulation may be the cause for the pro-arrythmogenic effect.

1.13 Framingham risk assessment

TThere is universal appreciation that being male, advancing age, elevated blood pressure, tobacco use, dyslipidaemia and diabetes mellitus are major contributors to the development of CVD. The overall vascular risk increases synergistically when multiple risk factors are present.[316] The Framingham risk score for predicting CVD has demonstrated validity among Whites, African Americans, Europeans and Asians.[317] The current Framingham risk score was derived from a population of 5 000 individuals in the Framingham community, taking age, blood pressure, TC, HDLC and tobacco exposure into consideration.[318] A 10-year risk of CVD >20% demands aggressive management. However, in certain settings, the Framingham risk assessment may be inaccurate. Studies have shown that patients with SLE and rheumatoid arthritis have a higher than the estimated CV risk.[319] Long-standing rheumatoid arthritis patients had more

coronary artery calcification and higher Framingham risk scores, with the absolute risk underestimated by the Framingham risk score calculation alone.[320] Diabetes is now recognised as a secondary prevention equivalent.[321]

1.14 Normative lipid data for South Africa

South Africa has a heterogeneous population in which different communities display not only different CVD event rates, but also varying lipid profiles.[322] 10-20 years ago, a number of localised population-based studies were conducted but these did not consider the effect of substantial urbanisation among the black population and the changing lifestyles of many South Africans. South Africa is burdened by the HIV epidemic. This adds further CV risk by the deranged metabolic profile, associated with highly active antiretroviral therapy. The dyslipidaemia associated with antiretroviral therapy manifests as a high triglyceride/low-HDLC syndrome, and in severe cases may present as a chylomicronaemia syndrome.[323] Current lipid profiles and CVD risk in different communities in South Africa are not available for specific comparisons with Addison's disease subjects in this study. By assuming that European and North American data apply,[302 324] risk estimates can be used as a guide, even if they are not absolutely reliable.

1.15 Dyslipidaemia and cardiovascular risk in Addison's disease

Dyslipidaemia may be heterogeneous in origin in patients with Addison's disease. Incidental lipid disorders can occur that are unrelated to Addison's disease, for example: familial combined hypercholesterolaemia (2% of any population) or familial hypercholesterolaemia (0.2% of most populations), but some founder effects exist in the South African population for familial hypercholesterolaemia.[325] Incidental disease states, such as nephrotic syndrome, hypothyroidism or diabetes may produce dyslipidaemias. The underlying aetiology of Addison's disease may also result in lipid derangements. Tuberculosis may cause an acute

phase reaction with reduced HDLC and by contrast, chronic tuberculosis may induce a rise in HDLC as patients are treated with rifampicin. Similarly, other microsomal enzyme inducers including: phenytoin and alcohol are known to raise HDLC (Table 4).[326]

Table 4: Possible causes for lipid derangement among patients with Addison's disease

	Primary	Secondary
Incidental causes of dyslipidaemia	• Familial combined hypercholesterolaemia • Familial hypercholesterolaemia • Dysbetalipoproteinaemia	• Nephrotic syndrome • Hypothyroidism • Diabetes mellitus
Related to underlying aetiology of Addison's disease *	• Autoimmune diabetes or primary hypothyroidism	• Tuberculosis acute or chronic or its therapy (rifampicin increases HDL) • Adrenoleukodystrophy
Related to Addison's disease and/or its treatment	• Glucocorticoid insufficiency (increased HDL)	• Glucocorticoid excess

* Conditions that may cause Addison's disease and affect other organ systems that modulate lipoprotein metabolism

Addison's disease has not traditionally been viewed as an independent CVD risk factor, and lipids and lipoproteins have not been studied in any depth in this condition. A single study examined lipid profiles of 54 Addison's patients in response to DHEA supplementation. The baseline and standard error of the mean (SEM) TC was 4.45 (0.22) mmol/L, TG was 1.23 (0.14) mmol/L, HDLC was 1.27 (0.08) mmol/L and LDLC was 2.57(0.15) mmol/L, and no differences in lipid profiles were found between the placebo and DHEA treatment groups after 6 months and 12 months.[241] In a recent study by Giordano et al, 18 of the 38 Addison's disease patients were hypercholesterolaemic and 18 patients had

hypertriglyceridaemia, compared to 8 controls, but none of the patients or controls had an HDLC <1.03 mmol/L or an LDLC >4.91 mmol/L.[240] It is conceivable that patients with Addison's disease have lipid abnormalities, which place them at increased risk for CVD and the GC replacement may also impose additional CVD risk. Patients with Addison's disease may also have altered markers for CV inflammation that result from the disease or its treatment, and the value of these markers needs to be assessed.

1.16 Therapy for Addison's disease

Patients with Addison's disease require lifelong GC replacement therapy. This usually takes the form of hydrocortisone. Endogenous cortisol production, determined by stable isotope dilution mass spectrometry in healthy individuals, is only 6-11 mg/m^2/day,[327] but the ideal hydrocortisone replacement dose remains to be determined. Suggested doses vary from 30 mg of hydrocortisone to as little as 12.5 mg per day, divided in two or three daily doses.[322 329] In general, clinicians rely on empiric doses, which vary substantially according to the practice at a particular centre. Over-replacement with hydrocortisone may result in accelerated bone loss, premature atherosclerosis and the metabolic syndrome.[235 330-332] On the other hand, insufficient hydrocortisone supplementation results in chronic symptoms of fatigue. There is evidence to support the idea, that irrespective of the dose of hydrocortisone replacement, patients have subjective impaired health quality.[333]

Conventional hydrocortisone replacement is non-physiological, as it is well absorbed in the small and large intestines, inducing high peaks that are followed by rapid elimination. This results in intermittent low trough levels require two to three doses per day. In addition, inter-individual differences in the absorption and metabolism of hydrocortisone, results in uniform doses being inappropriate for every patient with Addison's disease.[334] Irrespective of the modality of GC

replacement, normal physiology is not restored.[333] Consequently, there is ongoing research to improve hydrocortisone administration and replacement, by prolonging its bio-availability.[335]

CVD has been linked to increased cortisol levels. Hypertension, increased heart rate, increased TC, LDLC, fasting insulin and glucose, have all been reported to correlate with endogenous cortisol levels. A positive correlation has also been found between the number of carotid atherosclerotic plaques, measured by ultrasonography and endogenous cortisol exposure in an elderly population, independent of the number of coexisting CVD risk factors.[336] There is uncertainty as to the degree of either exogenous or endogenous cortisol exposure that results in harmful effects. In patients with hypoadrenalism, the most significant barrier to monitoring therapy with GCs is that no single test exists that reliably reflects adequacy, over-replacement or under-replacement.

1.16.1 Pharmacokinetic principles of monitoring and interpretation of drug levels

A priori assumptions are made with respect to drug monitoring, namely that there is inter-individual variation and that the drug levels found in plasma correlate much more closely with the therapeutic effect and toxicity than the dosage. For many drugs, therapeutic effects are not easily measured and utilising blood concentrations may be helpful in making dosage adjustments.[337] The standard definitions used in pharmacokinetic studies are given in Table 5.

After a patient ingests a single oral dose of medication, the peak level is referred to as the maximum concentration, C_{max}, or the maximum systemic exposure. The time at which C_{max} occurs is called tmax or the time of maximum exposure. Both C_{max} and t_{max} are highly dependent on how quickly the drug enters and is eliminated from the body. There is individual variation in the absorption, metabolism, utilisation and the elimination of drugs, which can be influenced by age, body

weight, gender, general state of health, genetic factors and drug interactions.[337] Drug concentrations may be measured in certain circumstances in whole blood, serum saliva or urine. The specific timing of the sample is likely to influence the interpretation of the drug concentration. The anticipated response of a drug is generally based on the concentrations determined by steady state samples or samples taken at specific time points after the dose. While hydrocortisone has excellent bioavailability after being administered orally, it has a half life of 1.8 hours and therefore does not reach a steady state using 2-3 daily doses.[338]

Drugs with a narrow therapeutic window (the blood concentration between the minimum effective and the maximum tolerated concentrations), require monitoring and dosage adjustments, in order to limit toxicity. For example, dosage adjustments for the aminoglycosides should ensure that the peak concentration is exceeded, while the trough concentration is below the threshold for toxicity.[339] The optimum therapeutic blood concentration range or therapeutic window in which most people will be effectively treated without suffering from toxicity, have been determined for many drugs, for example digoxin and lithium carbonate.[337]

1.16.1.1 Exposure-time profiles

The overall exposure to a drug is measured by the area under the concentration-time profile (AUC), also known as a systemic exposure-time profile, which is a composite of the rate and extent of drug input, distribution and elimination (Figure 9). AUC is derived from concentration time data. It takes cognisance of how much of the drug is introduced and cleared from the plasma compartment, irrespective of the rate at which it is absorbed into the plasma compartment. The usual way to determine AUC is by approximating the area between the measurements as trapezoids and summing up the areas of successive trapezoids. AUC may also guide dosing, for example, it may provide information on the maximum tolerated exposure and it is frequently referred to as AUC dosing. Drug AUC values can be used to derive other pharmacokinetic parameters, such as clearance or

bioavailability.[337]

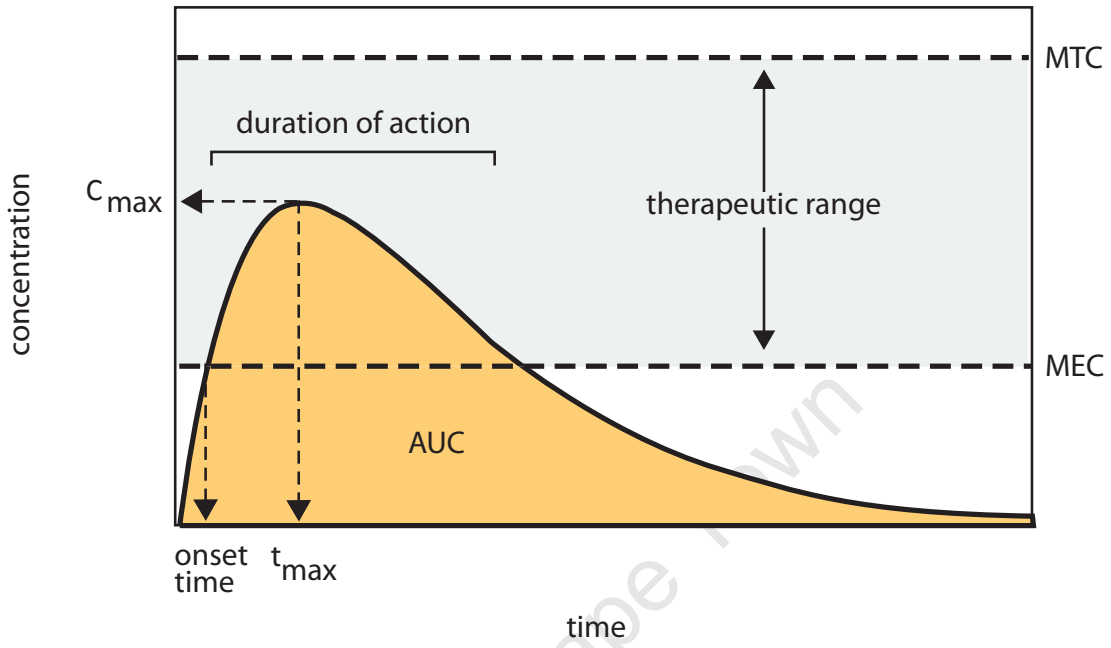

Figure 9: Pharmacokinetic data derived from an exposure time profile. The AUC (area under the concentration-time) correlates with exposure to a substance. The therapeutic window or therapeutic range is the optimal concentration at which is efficacy is most likely. The lower limit minimum effective concentration (MEC) is usually the concentration at which 50% of subjects have a therapeutic response. The upper limit minimum toxic concentration (MTC) is usually the lowest concentration at which the toxic effects predominate. The C_{max} (maximum concentration) of a substance is usually the highest total concentration of a drug or endogenous substance in plasma measured over a period of time. For an oral drug where the absorption into the plasma from the gut and clearance from the plasma via the kidney or liver compete, the maximum concentration, C_{max} occurs when these rates are equivalent. The t_{max} (time) after administration of the drug occurs when the maximum concentration is reached following an extravascular dose. Adapted from Rowland M, Tozer T. Clinical pharmacokinetics; concepts and applications. Third edition.San Francisco: Williams and Wilkens; 1995.[337]

Table 5: Standard definitions of pharmacokinetic data

Variable	Definition
Peak levels (C_{max})	The maximum concentration of a substance, measured during a defined interval. (For an oral drug, absorption processes increase the plasma concentration; distribution to other tissues from the plasma compartment and clearance from the plasma via the kidney and liver reduce plasma concentrations; the maximum concentration, C_{max} occurs when these rates of drug transfer into and out of the plasma compartment are equivalent.)
t_{max}	The time after administration of the drug when the maximum concentration is reached following an extravascular dose. More simply: the time of C_{max}
Trough levels (C_{min})	The minimum concentration observed over a period of time. (Usually this is the concentration in the plasma at the time just before the following dose, but can refer to any point in time when the concentration is lowest as in the case of endogenous substances.)
Area under the curve (AUC)	Area under the concentration versus time curve; usually the plasma drug concentration curve and this correlates with exposure to a substance. The measure will vary according to the method of derivation.
Therapeutic window	The therapeutic window or therapeutic concentration range is the optimal concentration at which is efficacy is most likely. The lower limit is usually the concentration at which 50% of subjects have a therapeutic response. The upper limit is usually the lowest concentration where the toxic effects predominate. Many prefer the term 'target drug concentration range' and this would be that concentration range where most patients would have a satisfactory treatment response without unacceptable toxicity.
Clearance (CL)	Total elimination of a substance from the body either by diffusion, excretion, renal filtration or metabolism (this would be referring to CL from the body). Often one mechanism of elimination predominates and therefore clearance refers to the elimination via that organ – for example kidney or liver. This is usually defined in terms of the volume of plasma (or defined compartment/tissue/body) cleared of the drug per unit time.
Bioavailability	The fraction of substance dose which reaches the systemic circulation. The lost fraction is the result of incomplete absorption and/or pre-systemic metabolism.

Adapted from Clinical pharmacokinetics: concepts and applications Rowland M, Tozer TN. 1995 Williams & Wilkins San Francisco Third edition.[337]

1.17 Monitoring of the adequacy of replacement in Addison's disease

A number of measures have been used to examine the adequacy or otherwise of replacement therapy in Addison's disease; none is ideal. These methods will be briefly discussed.

1.17.1 Use of plasma ACTH as a marker of hydrocortisone adequacy in primary hypoadrenalism

ACTH under normal physiological conditions is released in a pulsatile fashion, under control of hypothalamic corticotropin releasing hormone. The diurnal rhythm of ACTH release ensures that there is a peak before awakening and a continued decline as the day progresses. The episodic release of ACTH is independent of circulating levels of cortisol, and is not related to the levels of cortisol just prior to its release. It has a short plasma half life of 7-12 min inducing a wide range of concentrations of cortisol.[161]

The studies that utilise plasma ACTH as a marker of the adequacy of GC replacement in primary hypoadrenalism have yielded highly variable results. Feek et al showed that the morning plasma ACTH in Addison's disease patients, measured on the day that they were asked to omit their doses (equivalent to a total daily dose 20 mg daily of hydrocortisone) was either elevated, normal or suppressed.[340] In five healthy Addison's patients, presumed to be on adequate hydrocortisone replacement therapy (ranging from 15-30 mg daily), the ACTH was markedly elevated between 08h00 and 09h00, but was similar to control levels between 12h00 and 02h00.[341] On the other hand, it has been suggested that ACTH suppression below the reference range is indicative of over-exposure to GCs.[342] One of the explanations for the frequent lack of ACTH normalisation with replacement doses of GCs, is the reduced sensitivity of the pituitary gland to cortisol inhibition, which stems from the loss of negative feedback before the diagnosis and during treatment.[340] Thus unlike primary hypothyroidism, in which

TSH (which has a half life of 50-60 min and is under feedback inhibition of thyroid hormone) is accepted as a marker of adequacy of thyroxine replacement,[161] ACTH should not be used to monitor adequacy of GC replacement. It could also reflect the differences in the robustness of TSH and ACTH assays as well as stability of the hormone following sampling, since ACTH is more labile than TSH.

1.17.2 24-hour urine cortisol for the monitoring of hydrocortisone replacement therapy

The 24-hour urine cortisol measurement has limited value in monitoring and adjusting therapy for patients with Addison's disease. It is a gross reflection of hydrocortisone, because the peaks are ignored and it merely reflects a measure of total exposure.[343] In one small study of nine patients with primary hypoadrenalism on usual replacement doses, the urinary free cortisol (UFC) levels were in the range obtained from a large number of healthy controls.[344] However, in another study, the UFC levels were above the reference range.[343] These discrepant findings attest to the poor reliability and reproducibility of this modality for monitoring. Additionally, cortisol-binding globulin (CBG) is saturated rapidly after absorption of GCs. This leads to enhanced renal elimination of GCs, resulting in higher concentrations of UFC compared to healthy subjects, which makes it difficult to compare with the usual reference range.[345] Overall, the 24-hour urine collection has multiple drawbacks, including accuracy of urine volume collections, making it an unsuitable method of monitoring adequacy of replacement therapy.

1.17.3 Plasma cortisol for the monitoring of hydrocortisone replacement therapy

A variety of parameters utilising plasma concentrations have been reported.

1.17.3.1 Single measures of plasma cortisol-peak

Monitoring hydrocortisone replacement therapy using cortisol peak levels have revealed that the vast majority of patients have supra-physiological peak levels, occurring 1-2 hours after the morning dose, followed by hypocortisolaemia.[346] Li Voon Chong et al showed that the peak cortisol measured 2 hours after the morning dose was higher in patients taking 20 mg of hydrocortisone compared with those taking 10 mg.[329] Children with ACTH deficiency on hydrocortisone replacement had persistent supra-physiological peaks of cortisol, irrespective of whether the morning dose was adjusted for body surface area.[346]

1.17.3.2 Plasma cortisol day curves

Plasma cortisol day curves were first introduced in 1977. Plasma samples drawn hourly over a 5-hour period showed considerable inter-individual variation at both peak and 5-hour levels.[347] In 1978, patients on twice daily hydrocortisone were found to exhibit higher plasma concentrations between 08h00 and 12h00, and between 17h00 and 18h00 compared to control levels but plasma cortisol levels were undetectable between 03h00 and 08h00.[341] A three-times daily regimen of hydrocortisone was able to partially correct the prolonged nadir of plasma cortisol during the cortisol day curve seen with the twice-daily regimen.[334] Howlett et al performed serum cortisol measurements at 09h00, 12h30 and 17h30 on hypoadrenal patients' usual hydrocortisone replacement doses with the optimum replacement dose defined as the dose required to achieve a urinary free cortisol (UFC) and a 09h00 plasma cortisol level within reference range for the normal population. It was also revealed that following the peak cortisol levels, there are 5-7 hours of decline to below 100 nmol/L, which lasts until the next dose.[348] The aim of a structured scoring system was to assess the clinical quality of replacement together with timed repeated plasma cortisol measurements. In this system fatigue or loss of energy, nausea, weight loss, hyperpigmentation, hypoglycaemia, hyponatraemia or hyperkalaemia suggested under-replacement, while insomnia, recurrent infections, increased appetite, weight gain, glucose, hyperglycaemia,

hypernatraemia, hypokalaemia and elevated blood pressure suggested over-replacement. Lower scores correspond with lower plasma cortisol concentrations and higher scores correspond with with higher plasma cortisol concentrations[343] suggesting that a positive relationship exists between clinical parameters and cortisol concentrations during a plasma cortisol day curve. Peacey et al found that plasma cortisol day curves correlated with UFC levels and bone turnover markers in Addison's disease and hypopituitarism. At the beginning of the study, the mean total hydrocortisone dose was 29.5 mg and markers of bone turnover were increased. When the total dose of hydrocortisone was reduced to a mean of 20.8 mg in order to limit the harmful effect of GCs on bone, osteocalcin increased, but N-telopeptide of type I collagen (NTX) remained unchanged. [349]

Plasma cortisol day curves have inherent problems. The major drawbacks are the frequent blood sampling and admission to hospital, even for part of a day, resulting in inconvenience to the patient, and therefore these curves are not widely used. In addition, plasma cortisol relies on the hydrocortisone being absorbed systemically, equilibrating with CBG and does not reflect an instantaneous measure of free cortisol.[350] CBG levels vary widely among individuals[351] and may increase with increased duration of treatment with hydrocortisone, which may also contribute to the poor reliability of using plasma cortisol day curves.

1.17.3.3 Plasma cortisol day profiles and area under the curve

The AUC has also been used to estimate cortisol exposure. Mah et al showed that during the time period 08h00-14h00, it was possible to adjust the hydrocortisone dose so that the AUC was similar for patients and controls. Further, profiles were less variable when using a dose of 0.12 mg/kg of hydrocortisone and a single blood sample taken 4 hours after the initial dose of hydrocortisone could predict the AUC, which could be used to alter replacement doses.[350] Thomson et al reported significant inter-individual variability in plasma cortisol AUC, measured 15 min, 30 min, 60 min, 90 min, 120 min, 240 min, 360 min and 480 min after hydrocortisone

ingestion in patients.[352] Charmandari et al showed that using plasma AUC in patients with congenital adrenal hyperplasia, despite a high bioavailability with oral hydrocortisone, intravenous hydrocortisone induced a significantly higher C^{max} at 10 min, compared to oral hydrocortisone, which reached its peak cortisol concentration at 180 min. In both cases serum cortisol was undetectable 6 hours after the initial administration of hydrocortisone.[353]

1.17.4 Other biochemical markers for evaluating cortisol exposure

Remarkably, long-term cortisol exposure has been examined using the cortisol content of human hair as a biomarker. It is not a routine test, but it may in time become a successful, non-invasive alternative to salivary and plasma cortisol.[354]

1.18 Salivary cortisol as a means of monitoring replacement therapy in Addison's disease

Monitoring salivary cortisol will be introduced followed by a discussion on technical factors and previous studies of salivary cortisol in Addison's disease.

1.18.1 Introduction

There are numerous advantages to using saliva for monitoring drugs or hormones. Saliva is readily accessible, it is a relatively small reservoir for antigens and as a result, the risk of contracting HIV and hepatitis infections is much lower than for blood. Analytes are stable at ambient temperature for about 7 days, limiting the need for refrigeration. It is more convenient for patients than plasma samples, as multiple samples can be collected at their homes and it does not require supervision by a professional.[355]

Salivary cortisol is a non-invasive accurate measure of endogenous free cortisol production and it has found favour in various fields such as sports physiology, endocrinology, immunology and psychology.[356] The steroids enter the saliva

through the acinar cells of the salivary glands and the rate at which they are found in saliva is independent of the amount of salivary flow.[355 357] However, minor abrasions of the gum mucosa can induce contamination by blood, which could falsely elevate salivary cortisol.[355] Steroids are lipophilic and are transferred rapidly through the saliva. A significant proportion of cortisol is converted to cortisone by 11 beta-hydroxysteroid-dehydrogenase-II, yielding an inactive keto-form. Hirasawa et al confirmed immunoreactivity of both 11 beta-hydroxysteroid-dehydrogenase-II and mineralocorticoid receptors in submandibular and parotid salivary glands.[358] The degree to which this enzymatic reaction occurs is often ignored, which could potentially explain some of the discrepant results in the literature with respect to cortisol. In order to obviate this uncertainty, it is important to verify that the assay used has minimal cross-reactivity with cortisone.[359] As there is comparatively minimal 11 beta-hydroxysteroid-dehydrogenase-I in salivary glands, salivary cortisone predominates over salivary cortisol from local production and salivary cortisol has been suggested to be a good reflection of serum cortisol.[360 361]

Salivary cortisol correlates well with an individual's degree of stress and socio-economic status. In two groups representing divergent socio-economic classes, the lower socio-economic status group had a higher stress index and salivary cortisol, compared with the higher socio-economic class.[362] In a pilot study in young males with violent and aggressive behaviour, salivary cortisol was reproducible from week to week, implying that a single day curve may be of value in assessing the HPA.[363] Salivary cortisol could be considered an ultra-filtrate of plasma cortisol as saliva is usually free of CBG and thus measures free cortisol.[364] Salivary cortisol has specific advantages over plasma cortisol in pregnancy and patients using oral contraceptives or hormonal replacement therapy, where the CBG levels are likely to be elevated. Salivary cortisol has also been extensively used to exclude Cushing's syndrome, and more recently it has been employed to diagnose hypo-adrenal patients.[365 366] Additionally, salivary

cortisol can be used in basal and stimulated conditions, as it does not require equilibration with CBG.[367] Salivary cortisol has emerged as an important analyte to monitor conditions ranging from frank excess, example Cushing's disease to subtle degrees, of HPA activation or depression.[368]

Several studies have shown a correlation between salivary cortisol and several metabolic abnormalities.[369 370] A rise in salivary cortisol was positively associated with BMI, waist-to-hip ratio, random blood glucose (RBG), insulin and TG, and with abdominal obesity in men.[368] Among type 2 diabetics, women with the highest cortisol profiles had the highest fasting, postprandial glucose and HbA1C. In addition, systolic and diastolic blood pressures were correlated with cortisol levels.[371]

Multiple authors have shown an association between cortisol hyper-secretion and the development of clinical depression.[372 373] People at risk of depression due to a positive family history have demonstrated increased salivary cortisol levels. In keeping with a previous finding of a negative correlation between plasma cortisol levels and bone mineral density (BMD), similarly increased salivary cortisol levels and reduced BMD at all sites were demonstrated in depressed individuals.[374] By contrast, in elderly women from Helsinki, salivary cortisol samples throughout a 24-hour period did not demonstrate a relationship between HPA activity and the development of the metabolic syndrome.[375]

Although a positive correlation between salivary cortisol and serum cortisol exists in most studies, correlation coefficients vary from $r = 0.62$ to $r = 0.86$.[376 377] An overall correlation of simultaneous measures of serum with salivary cortisol was found to be $r = 0.62$; $p=0.0001$ in healthy subjects, and patients with Addison's disease, pseudo-Cushing's and Cushing's syndrome. Wong et al, found that total plasma cortisol was highly correlated with salivary cortisol ($r = 0.7$) in hypoadrenal patients on hydrocortisone replacement therapy.[376] The possible effect of variation

in CBG (peak in the early afternoon and attenuation of free cortisol) and variable binding of cortisol, due to diurnal variation may be potential causes for the variable relationship between salivary and serum cortisol.[378] Healthy subjects' morning serum cortisol in one study is about 20 times greater than that of simultaneously sampled salivary cortisol, and in the afternoon serum cortisol is about 27 times greater than salivary cortisol.[379] Despite the multiple influences that could modify the relationship between serum and salivary cortisol, one study demonstrated an excellent correlation throughout the 24-hour period.[380] Overall, the relationship between saliva and serum cortisol is not always constant and is influenced by the time of sampling.

1.18.2 Devices for the collection of salivary cortisol

Saliva is most commonly collected by chewing on salivettes or by collecting passive drool in plastic tubes. It has been reported that the concentrations of salivary cortisol generated, were lower when collected in salivettes, compared with the passive drool technique collected into microcentrifuge tubes. However, salivettes exhibited better correlation with total plasma and free plasma cortisol compared to collecting saliva using the passive drool technique.[380] Similarly, when using 6 time points between 08h00 and 23h00, the salivettes correlated better with plasma free and total plasma cortisol compared to the passive drool technique.[381] As results may be influenced by the particular collection device, the same device should be used throughout studies.[382]

1.18.3 Technical factors associated with salivary cortisol collection

One of the problems with saliva analysis is that the steroid of interest may coexist with a difficult matrix, requiring physical or chemical disruption. Freezing and thawing cycles, and centrifugation are used to break up mucins.[383] A significant decrease in the concentrations of salivary cortisol were detected in the samples that were thawed and refrozen up to five times, but thawing twice did not induce significant differences.[384] Blood in the saliva due to brushing teeth, use of oral

tobacco or gingivitis may falsely elevate salivary cortisol. A useful method of determining whether saliva is contaminated by blood, is to quantify the volume of sex-hormone binding globulin (SHBG) and CBG.[385] Exogenous GCs may also contaminate saliva.[383] Many authors have confirmed the stability of salivary cortisol for up to 3 months at -20°C. Storage at -80°C may prolong the durability of the samples even further. An increase in cortisol in response to chronic smoking has been reported and it is thought to be the result of activation of the HPA. Smokers exhibited higher levels of salivary cortisol throughout the day, despite being carefully controlled for social status, health behaviour and stress reporting in one study.[386] The collection process for salivary cortisol also demands some dietary restrictions as certain chemicals may influence the measured cortisol concentrations.[387] For example, lemon juice intended to stimulate saliva may cause a false increase in salivary cortisol.[388]

An advantage of using salivary cortisol is it can be performed in the comfort of patients' homes, rather than requiring admission to hospital, obviating spurious rises in levels of cortisol associated with stress caused by being admitted to hospital. Sheer et al showed that the salivary cortisol on awakening was two times higher in subjects admitted to hospital, three times higher at the morning peak and five times higher at late night sampling, compared to those subjects who collected saliva at home.[389]

1.18.4 Monitoring of hydrocortisone using salivary cortisol in Addison's disease patients

Despite salivary cortisol being easily obtained, there are concerns relating to its wide variability. Two authors have suggested that measuring salivary cortisol is not of value in assessing GC adequacy, because of the significant variability observed.[352 376] Free cortisol levels fluctuate considerably during the day, suggesting that salivary cortisol may be no more accurate than plasma cortisol in assessing hydrocortisone replacement[352] (Table 6).

Table 6: Previous studies of salivary cortisol in Addison's disease

Year	Age (years)	*N*	Addison's Disease/ hypopituitarism	Methods	Findings	Reference
2008	51 Median	11	Addison's Disease on hydrocortisone replacement therapy But omitted hydrocortisone replacement therapy for 24 hours	Salivary cortisol was measured at 08h00 and 00h00	• Serum and salivary cortisol, were highly correlated r = 0.62, p = 0.0001 • Salivary cortisol levels were lower in patients with Addison's disease compared to controls 4.14 versus 18.5 nmol/L	Restituto 2008[390]
2007	44.7 Median	5	Addison's disease patients on continuous subcutaneous hydrocortisone infusion	Salivary cortisol samples were taken at 07h00, 09h00, 11h00, 13h00, 19h00, 21h00 and 23h00	• 7 samples were taken between 07h00 and 23h00 • Significant variability noted • Large proportion of the salivary cortisol samples were supra-physiological	Lovas 2007[391]
2006	60 Median	31	Addison's disease , correlation studies were performed after intravenous and oral hydrocortisone	After 12.5mg cortisone acetate taken at 08h00, salvary cortisol was measured at 09h30, 11h00, 12h30 and in another sub-group of patients were evaluated on their usual doses.	• Excellent correlation between saliva cortisol and serum r = 0.86, $p < 0.002$ • Excellent correlation between salivary cortisol and serum after intravenous hydrocortisone r = 0.95-0.98	Lovas 2006[377]

Year	Age (years)	*N*	Addison's Disease/ hypopituitarism	Methods	Findings	Reference
2007	5.1-18.5 (age range)	30	Children and adolescents with hypopituitarism on hydrocortisone replacement	Salivary cortisol samples were taken at 08h00, 10h00, 12h00, 14h00, 16h00, 18h00, 20h00	• Salivary and serum cortisol were sampled between 08h00 and 21h00 • Correlation between saliva and serum not as good as between blood spot and serum cortisol	Maguire 2007[346]
2004	49.8 Median	18	Addison's disease and hypopituitarism. Samples were collected at seven predetermined time points	Salivary cortisol was sampled before the morning dose of hydrocortisone, 1 hour after the first dose, 2 hours after the first dose, before the lunch dose, 1 hour after the lunch dose, 2 hours after the lunch dose and before dinner dose	• Salivary cortisol correlated with serum cortisol $r = 0.63$, $p < 0.001$ • 49% of the salivary cortisol levels were above normal range and 2% were below the normal range.	Wong 2004[376]
2007	47 Median	27	Addison's disease or hypopituitarism salivary cortisol measured after intravenous hydrocortisone and then after the usual oral hydrocortisone dose	Salivary cortisol sampled hourly for 8 hours after hydrocortisone dose	• Maximum salivary cortisol concentrations range from 21 to 2000 nmol/L observed 0.25-2 hours after the oral dose of hydrocortisone • Salivary cortisol results were highly variable • Salivary cortisol AUC failed to predict plasma cortisol AUC ($r^2 = 0.16$)	Thomson 2007[352]

References in superscript

N: number

Løvås K et al evaluated the potential for using salivary cortisol measurements in assessing the adequacy of GC replacement in patients with Addison's disease, after taking either oral cortisone acetate or intravenous hydrocortisone. Salivary and plasma cortisol were highly correlated, ($r = 0.83\text{-}0.98$, $p < 0.002$), but a morning dose of 12.5 mg of cortisone acetate generated a wide distribution in salivary cortisol concentrations.[377] Salivary cortisol AUC has a poor predictive value for plasma cortisol AUC and consequently, it was suggested that salivary cortisol could not be used to make individual adjustments to hydrocortisone doses.[352] Hydrocortisone is the favoured GC used in congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency, due to its short half life and neutrality with respect to longitudinal growth.[392,393] The usual profile after taking oral hydrocortisone is that the peak plasma cortisol is reached 1-2 hours after administration in the morning, followed by a non-exponential decline indicating that a non-sustained level of cortisol prevails after taking oral hydrocortisone.[239] Groschl et al examined the pharmacokinetic profile of a single dose of hydrocortisone in ten children and adolescents with salt-wasting CAH and compared it to that of healthy volunteers, aged between 18 years and 29 years of age. The patients with CAH had different cortisol profiles from the healthy volunteers; as they reached a maximum salivary cortisol concentration within 15 min after taking oral hydrocortisone, while the healthy volunteers reached their maximum concentrations only between 15 min and 30 min. This suggests that the metabolism of hydrocortisone is rapid in patients with CAH, due to lower levels of CBG and consequently accelerated clearance rates. Although it was unclear whether the BMI and the dose of hydrocortisone/m2 differed between the patients and controls to account for the differential clearance rates in these two groups it is also uncertain, whether this rapid rate of clearance occurs in Addison's disease.[392]

1.19 Improvements with respect to hydrocortisone replacement therapy

Experts have suggested hydrocortisone doses should be between 20 mg and 30 mg per day, divided in two or three daily doses.[348] An intravenous programmed hydrocortisone infusion was set up to mimic the circadian rhythm of cortisol secretion. The overall mean 24-hour cortisol exposure was similar for the patients on conventional oral therapy and infusion therapy, but the patients on infusion therapy were exposed to higher concentrations between 21h00 and 09h00. These results indicate the potential to improve therapy, by focussing on delayed absorption.[394] Modified hydrocortisone release tablets administered 15-20 mg at 23h00 and 10 mg at 07h00, provided the most physiological excursions of cortisol, compared to healthy controls.[395] Another hydrocortisone formulation administered to healthy subjects, providing 15 mg with a 50% delay in release, was able to match normal cortisol profiles.[396] A novel once daily modified release hydrocortisone tablet with combined immediate and extended release characteristics has been developed to achieve a more physiological cortisol profile reproducing the normal circadian profile better than immediate release hydrocortisone. The pharmacokinetic profiles indicate that the gastrointestinal absorption was rapid with a high bioavailability and the variability was less than indicated by immediate release hydrocortisone formulations. This preparation has completed phase III studies.[397] Overall, the benefits seen with the delayed release are the lower peak levels and the absence of sub-physiological cortisol levels as seen using conventional replacement modalities.

1.20 Monitoring of salivary cortisol, using the area under the curve

The AUC for salivary cortisol is a useful surrogate measure of exposure to either endogenous or exogenous cortisol. In chronic fatigue sufferers and healthy controls, the salivary cortisol AUC did not differ, but the first hour AUC of the

former was lower compared to the healthy controls.[398] Inhaled GCs have the potential to suppress the HPA and despite the recommendation for frequent monitoring of serum cortisol levels, the ideal modality for monitoring has not been established. 153 patients with moderate asthma were randomly assigned to receive fluticasone propionate in varying doses, flunisolide or prednisone. An 08h00 salivary cortisol, 24-hour UFC and 22 hour serum cortisol AUC were performed. A positive correlation was found between the 08h00 salivary cortisol and serum cortisol AUC ($r = 0.51$; $p = 0.0001$), as well as with the serum cortisol performed at the same time, ($r = 0.55$; $p = 0.0001$). The salivary cortisol measured once at 08h00 showed a 50% decline in the plasma cortisol AUC for a 22-hour period.[399]

There is consensus that the salivary cortisol AUC was the most promising measure in order to link cortisol levels and psychological functioning. Increased salivary cortisol AUC concentrations have been related to psychosocial measures for example anxiety, hostility and lack of calmness. Stone et al suggested that in order to evaluate exposure, more salivary cortisol measures per day are better than fewer[400] and at least four or five are recommended. To obtain an accurate AUC samples taken 1 hours, 4 hours, 9 hours and 11 hours after waking have been shown to provide reasonable assessment of the AUC. Other researchers have recommended six samples taken at immediately on waking, before getting out of bed, 45 minutes after waking, between 16h00 and 18h00, 18h00 and 21h00 and between 21h00 and bedtime. The frequent sampling, especially during the early part of the day, ensures that the morning cortisol awakening response (CAR) is adequately measured, that the afternoon-evening slope is demonstrated and finally that the evening nadir is confirmed.[401] A multidisciplinary collaboration of leading scholars examining socio-economic status and health, known as the MacArthur Network has suggested a single day, six-sample protocol with samples taken at awakening, 45 minutes, 2 1/2 hours, 8 hours and 12 hours after waking and at bedtime, in order to cover the troughs and peak

levels during waking hours.[402] Alterations in the CAR have been documented in disease states. Normally, the peak concentration occurs about 30 minutes after waking, but significant variability in the CAR has been encountered in breast cancer sufferers.[403] The normal circadian rhythm may be disrupted prior to the diagnosis of either diabetes or hypertension.[404] Obesity too is known to exhibit a blunted salivary cortisol response, with overall lower morning levels.[405]

An elevated CAR has been associated with social stress, depression and chronic work overload, while a reduced response can be anticipated with persistent pain.[406] The variation in the magnitude of responses between individuals, following different stressful situations or tests, is marked. In contrast to pharmacological stimulants, for example administration of ACTH or corticotrophin releasing hormone, psychological stress tasks have differing potential to evoke stress responses. The abundance of psychology literature has shown that certain individuals respond differently depending on whether the stressor is isolated with acute hyper-responsiveness or a recurrent insult, manifesting with hypo-responsivity. Inconsistencies in results may be attributable to the highly varied responses and heterogeneity of the study design.[407]

1.20.1 Hydrocortisone during stress and mineralocortioid replacement

At least a doubling of the dose of hydrocortisone is warranted in most mild illnesses. In life-threatening situations, the dose of hydrocortisone should be increased to 100 mg, three times daily. Major surgical procedures, sepsis or severe trauma demand significant escalation of hydrocortisone replacement, but this may be reduced gradually, depending on a favourable patient response. On the other hand, a Cochrane review examining the need for supplemental steroids for surgical patients with adrenal insufficiency, showed inadequate evidence to support or refute the use of supplemental peri-operative steroids in these patients and that the maintenance dose may be sufficient.[408] Mineralocorticoids are essential for the reversal of aldosterone deficiency. Assessment of mineralocorticoid adequacy

is possible by evaluating plasma sodium, potassium and renin activity assays. An elevated plasma renin activity in an Addison's patient may indicate inadequate mineralocorticoid substitution, while elevated blood pressure, peripheral oedema and sodium retention may indicate over-replacement. The usual dose of 9-alpha-flouro-hydrocortisone is between 50 μg and 200 μg per day. Patients are at risk of developing hyperkalaemia and hypotension, should this be discontinued.[229]

1.20.2 Rationale for measurement of salivary cortisol day curves in Addison's disease

Salivary cortisol is easily accessible and reflects both circulating free and total serum cortisol. Thus salivary cortisol day curves, could be used to determine whether Addison's patients on their usual doses of hydrocortisone are exposed to higher concentrations of cortisol than healthy subjects' endogenous cortisol levels and whether higher exposure translates into metabolic abnormalities.

1.21 The effect of glucocorticoid receptor polymorphisms on sensitivity to cortisol in relation to Addison's disease

1.21.1 Introduction

Cortisol exerts multiple pleotropic actions that are critical for metabolic, physiological and stress-related conditions.[409] The actions of cortisol are diverse, and include CV immune and metabolic actions, as well as regulation of cellular growth and proliferation. Cortisol also exerts negative feedback action on the HPA, which is fundamentally important in regulating its secretion. Multiple psychiatric and psychological disorders arise from over-activation of the HPA.[410] Adipocyte differentiation, for example which increased fat mass and predisposition to insulin resistance, is also influenced by cortisol. As cortisol has a permissive effect on catecholamines, increased lipolysis and circulating FFA levels may result during periods of physical and psychological stress, and supra-physiological GCs.[161,411,412]

The biological response to cortisol is influenced by the level of GCs and the sensitivities of the individual glucocorticoid receptors (GCRs).[413] The biological response is also influenced by the availability of prevailing cortisol and the extent to which cortisol is bound to CBG. 11 beta-hydroxysteroid-dehydrogenase-I converting enzyme determines the ratio of active versus inactive intracellular cortisol and is consequently capable of altering the biological response.[414] The response that an individual patient exhibits to either physiological or supra-physiological GCs is not uniform and may differ even within tissues of the same individual.[415] Similarly, the same stressor evokes different stress responses in different people, invoking the possibility of individual sensitivity to cortisol. Plasma cortisol levels do not invariably correlate with the functional effects on the target tissues, as considerable variability is observed among subjects using GCs in relation to therapeutic efficacy and development of side-effects.[416] On binding of GCs to their glucocorticoid receptor (GCR) apparatus, the GCR evokes biological changes on the target tissues. Multiple functional polymorphisms within the GCR may explain the differential sensitivities of patients to GCs.

1.21.2 The glucocorticoid receptor

Survival of the mammalian species is critically dependent on the GCRs, as shown by GCR gene knockout mice experiments in which these mice die soon after birth.[417 418] The GCR is a member of the steroid hormone receptor-binding super family and common to all these receptors are an N-terminal transactivating domain, a DNA-binding domain and a C-terminal binding domain[418] (Figure 10).

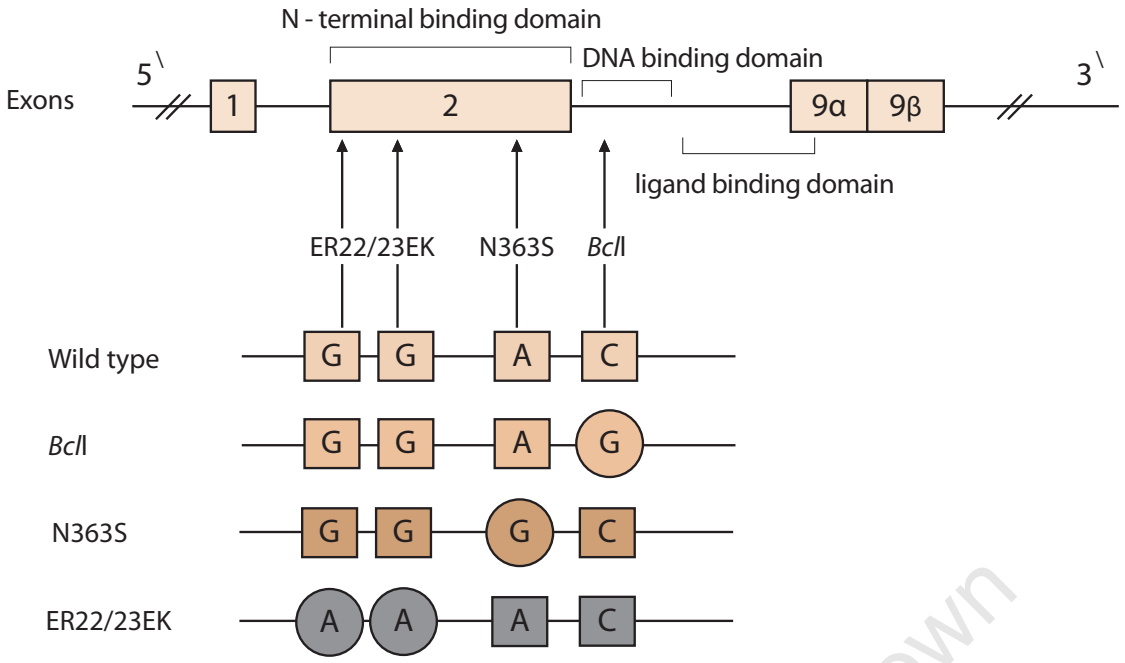

Figure 10: Schematic representation of three single nucleotide polymorphisms in the glucocorticoid receptor that have been examined in this study. Adapted from Koeijvoets KC, van der Net JB, van Rossum FC, Steyerberg EW et al, Two common haplotypes of the glucocorticoid receptor gene are associated with increased susceptibility to cardiovascular disease in men with familial hypercholesterolaemia. J.Clin. Endocrinol. Metab. 2008 93:4902-4908.[420]
A, C, G = DNA base changes.

The human GCR consists of nine exons, located on chromosome 5 (5q31) and is encoded by the gene nuclear receptor subfamily 3, group C, member 1 (NR3C1).[421] Alternative splicing of the GCR messenger ribonucleic acid (mRNA) produces two distinct products namely GCR-α and GCR-β. GCR-α and GCR-β both contain exons 1-8, but they differ due to alternative splicing of exon 9, which produces a 5.5-kb and 4.3-kb mRNA, respectively.[422] Several polymorphisms have been reported in the GCR gene, which occur in different regions and so affect the GCR function in different ways (Figure 10). The ER22/23EK polymorphism results in GCR reduced sensitivity, while both N363S and BclI polymorphisms lead to enhanced sensitivity. The altered sensitivity imparted by GCR polymorphisms is a consequence of a modified transcript, rather than a loss of transcript. Activation

of the GCRs occurs through the binding of cortisol and the translocation of this entire complex to the nucleus, where it binds to specific DNA sequences and activates transcription of a wide variety of GC responsive genes.[418 423]

When cortisol binds to GCR-α, an initial conformational change results, with consequent exposure of its nuclear localisation signal and subsequent nuclear entry, aided by the protein product, importin. Cis-regulation is achieved through direct interaction between GCR-α and DNA. By contrast, interaction between GCR-α and other transcription factors gives rise to transregulation.[415] GCs mediate their anti-inflammatory action through the transrepressive effects, which are the processes that inhibit cellular migration and humoral immunity, and transactivation enhanced production of Iκβ (specific inhibitor of transcription factor nuclear factor κβ) results.[424] When cortisol binds to the GCR, the activated GCR complex up-regulates the expression of anti-inflammatory proteins in the nucleus and may simultaneously suppress the pro-inflammatory proteins in the cytoplasm. This is achieved by preventing translocation of other transcription factors from the cytoplasm into the nucleus. Transactivation is a process by which the GCR homodimerises, translocates into the nucleus and then binds to specific DNA response elements, activating gene transcription. Each cell type may have any unique biological response to GCs. Transcription factors NF-κβ or AP-1 are able to transactivate various genes in the absence of an activated GCR. On the other hand, the activated GCR can bind with these transcription factors and prevent them from targeting specific genes. This process is called transrepression. Transactivation and transrepression may each have adverse and beneficial effects. Examples of adverse effects resulting from transrepression include HPA axis insufficiency and susceptibility to infections, while diabetes, glaucoma and skin atrophy result from transactivation adverse effects. The beneficial effects of GCs may also result from transrepression (for example, inflammatory gene inhibition, immune cell migration and suppression of carcinogenesis), and transactivation (for example up-regulation of anti-inflammatory lipocortin-1, secretary leukocyte peptidase inhibitor and MAP kinase phosphatase-1).[425]

The GCR-α is the most comprehensively studied protein product of the GCR gene family, because of its functional importance to the steroid binding activity.[426] The protein product GCR-β does not bind to either synthetic or endogenous GCs. Its principal action is to antagonise GCR-α and to modulate the cortisol response.[418] GCR-β exists in considerably lower concentrations than GCR-α consequently, low GCR-α: GCR-β ratios are thought to play a role in the development of GC resistance.[427]

The severest form of altered receptor function is generalised GCR resistance, arising from diminished transactivation of the GC-responsive mouse mammary tumour virus (MMTV) promoter.[428] This is a rare, familial or sporadic condition, in which the target tissues display either general or partial insensitivity to GCs and a compensatory increase in ACTH levels.[429] The clinical picture may vary from a complete lack of symptoms to overt clinical GC deficiency, despite elevated compensated plasma cortisol and ACTH levels. This excessive ACTH secretion results in adrenal hyperplasia, and increased production of adrenal steroids and mineralocorticoids.[428]

1.21.3 Glucocorticoid receptor polymorphisms

The marked variation in patients' clinical responses and susceptibility to developing GC side effects is well recognised, but there is debate as to whether this is modified by gender and age.[428 430-434]

Several GCR polymorphisms have been reported. The most common include: *Bcl*I, ER22/23EK and N363S, which have been investigated extensively in the context of metabolic syndromes. The *Bcl*I polymorphism is due to an intronic C-to-G nucleotide change that appears 646 base pairs (bp) downstream from exon 2 and involves a *Bcl*I restriction site in intron 2. The ER22/23EK is due to changes in codons 22 and 23 (GAG AGG to GAA AAG allele). The ER22/23EK polymorphism consists of two linked point mutations. The mutation in codon 22

is silent as the change from GAG to GAA still codes for glutamic acid (E to E), while the change in codon 23 AGG to AAG, changes the amino acid from arginine to lysine (R to K). The N363S polymorphism is found in exon 2, resulting in a change from asparagine to serine (N to S).[419] In addition to these functional polymorphisms, there are a large number of other polymorphisms that do not appear to affect function.[435]

The precise mechanism accounting for the increased sensitivity of *Bcl*I to GCs is not known. No alteration in GCR pre-mRNA has been identified, but it is speculated that this polymorphism is linked to variations in the promoter region, resulting in an increased expression or increased stability of the GCR gene.[436] Using binding studies with {(3)H}-dexamethasone, Russcher et al,[437] demonstrated that 15% more of a less active transcriptionally active isoform was produced by cells harbouring the ER22/23EK polymorphism. Using microarray gene technology, the N363S polymorphism was shown to regulate a different set of genes, which accounts for a differential effect of dexamethasone in cells harbouring this polymorphism.[437] Transfection experiments indicated that a number of genes are up-regulated in the presence of this polymorphism.[438] In addition, the N363S polymorphism induced a small increase in the transactivating capacity of the GC response element-luciferase reporter.[439]

1.21.3.1 Prevalence of glucocorticoid receptor polymorphisms

Ethnicity appears to have a major influence on the prevalence of GCR polymorphisms. Caucasian controls were selected and matched for asthmatics in Poland to determine whether an association exists between GCR polymorphisms and response to GCs. The frequency of the *Bcl*I (G allele) was 38% and the (C allele) was 62% in this Polish cohort.[440] Among Polish healthy control subjects, the frequency of the *Bcl*I (G allele) for homozygotes was 12.9% and 47.1% for heterozygotes.[441]

The reported frequency of the N363S (G allele) is 3-7% in Dutch, Anglo-Celtic and French Caucasian populations from Europe.[443-445] By contrast, a significantly lower prevalence of the N363S (G allele) was found in South Asians (0.3%) living in the United Kingdom (35% Indians, 42% Pakistanis and 19% Bangladeshis).[446] Among Brazilian subjects, the prevalence of the N363S (G allele) has been reported as 3.4%, while prevalence of up to 19% has been reported in an Australian population based study.[447] A recent Polish study of healthy control subjects, indicated that the G allele of the N363S polymorphism occurred at a frequency of 10%.[441]

There are only occasional studies in South Africa that examined the prevalence of these polymorphisms. In her dissertation in 2007, Jennings et al found that the prevalence of the *Bcl*I (G allele) polymorphism among normal weight and obese Black South African women subjects was 82% and 81%, respectively. The G allele of the N363S polymorphism to date has only been reported in the minority of black Africans, but as far as is known, this polymorphism has not yet been investigated in South Africa.[448] There is also no record of the ER22/23EK polymorphism being investigated in South Africa. See (http://www.ncbi.nlm.nih.gov/projects/SNP). Additional prevalences for the various polymorphisms in relation to ethnicity were obtained from the NCBI set of databases (Table 7).[449]

Table 7: The known frequencies of the three cardinal SNPs of the glucocorticoid receptor in different ethnic groups

	rs41423247 *BclI* (C to G)		**rs6189 ER22/23EK** (GAG AGG to GAA AAG) arginine to lysine		**rs6195 N363S** (A to G) asparagine to serine	
	Allele 1 C	**Allele 2 G**	**Allele 1 GAG AGG**	**Allele 2 GAA AAG**	**Allele 1 A**	**Allele 2 G**
Cauca-sians	0.33	0.67	0.97	0.03	>0.92	up to 0.08
Asians	unknown	unknown	0.997	0.003	>0.97	0-0.03
Blacks	unknown	unknown	0.998	0.002	0.975-1	0-0.025

Reference: http://www.ncbi.nlm.nih.gov/projects/SNP

A, C, G=DNA bases

SNPs single nucleotide polymorphisms

1.21.3.2 General anticipated effects of the three functional polymorphisms

The *Bcl*I (C to G) polymorphism has been studied in relation to metabolic and various inflammatory or autoimmune conditions, following the observation that the polymorphism may be associated with enhanced cortisol sensitivity.[448] The ER22/23EK (GAG AGG to GAA AAG) polymorphism has been reported to display decreased sensitivity to dexamethasone, with significantly greater levels of post dexamethasone cortisol, and it has also been studied in relation to metabolic profiles.[450] The N363S (A to G) polymorphism is associated with increased GC sensitivity following various studies of low-dose dexamethasone, which evoked increased cortisol suppression.[439 443]

1.21.4 Phenotypic differences conferred by glucocorticoid receptor (GCR) polymorphisms

Single nucleotide polymorphisms (SNPs) in different combinations give rise to

haplotypes, producing variations in phenotype. GCR polymorphisms may be associated with alteration in body composition and metabolic parameters,[433] which are not clinically easily recognisable thus the dexamethasone suppression test may be useful in assessing the clinical phenotype, defined by the concentration of early morning cortisol after administration of dexamethasone on the night before. The major problem with identifying the impact of the GCR polymorphisms on measurable variables such as glucose, lipids, lipoproteins and bone, is that these clinical parameters are affected by so many factors it is uncertain whether the GCR imparts different effects in different tissues.

1.21.4.1 Exogenous glucocorticoid response

It may be expected that individuals exhibiting increased central sensitivity to GCs through sensitising polymorphisms of the GCR that they may produce less total cortisol and suppress production to lower levels of cortisol. However, the basal cortisol levels in the morning prior to dexamethasone suppression were no different between the heterozygous or homozygous carriers of the *Bcl*I (C to G allele), compared with non-carriers.[436] Moreover, fasting cortisol levels prior to dexamethasone suppression were no different between N363S (G allele) and non-carriers.[443] Similarly, it could be expected that the subjects with the ER22/23EK (GAA AAG) polymorphism may exhibit increased basal cortisol levels to overcome a degree of resistance, yet these concentrations were no different between carriers and non-carriers.[450] It is, however, conceivable that the total daily cortisol production might be altered, whereas a single random fasting basal cortisol, may not be affected.

Dexamethasone is used to test for negative feedback inhibition of cortisol. Individuals harbouring either the *Bcl*I (G allele) or the N363S (G allele) polymorphisms,[443] have increased suppression of cortisol in the morning, which is compatible with enhanced sensitivity to GCs. Healthy subjects exhibiting the BclI polymorphism (G allele), have lower post-dexamethasone plasma cortisol,

corroborating the concept of increased sensitivity with exogenous GCs.[433] For the carriers of the *Bcl*I (G allele), both the 1 mg and 0.25 mg dexamethasone resulted in significant cortisol reduction, with heterozygotes exhibiting a mean absolute reduction of 10 nmol/L and homozygotes approximately 15 nmol/L in the former dose category, compared with the wild-type. The absolute difference in the latter dose category was approximately 50 nmol/L and approximately 75 nmol/L respectively.[436] By contrast, the ER22/23EK (GAA AAG allele) variant results in decreased sensitivity, as measured by higher post dexamethasone cortisol levels.[451] The absolute reduction in serum cortisol following 1 mg and 0.25 mg of dexamethasone in relation to the ER22/23EK polymorphism was 467.0 nmol/L compared with 484.5 nmol/L in non-carriers and 259.7 nmol/L versus 285.89 mmol/L in non-carriers respectively.[450] The N363S (G allele) polymorphism carriers had a mean absolute reduction of cortisol following a 0.25 mg dexamethasone suppression test of 373 nmol/L, compared with 280.5 nmol/L in healthy controls However, the 1 mg dexamethasone suppression test failed to demonstrate any difference in cortisol concentration.[443]

The sensitising effect of the N363S (G allele) polymorphism is further corroborated by Di Blasio et al, who reported that healthy carriers of the N363S (G allele) polymorphism exhibited increased sensitivity to low-dose (0.25 mg) dexamethasone suppression testing. This was particularly evident in overweight subjects whose BMI was <28 kg/m^2) and in those individuals with the N363S (G allele) polymorphism who had higher mean BMI compared to non-carriers.[452] A significant correlation between the N363S (G allele) polymorphism and prednisolone-induced ocular hypertension was found in patients undergoing a photorefractive keratectomy.[453] Duchene's muscular dystrophy patients exhibited a variable response to steroids, and in the analysis of the GCR polymorphisms, the N363S (G allele) polymorphism demonstrated a trend towards latest onset loss of mobility.[454] Szczepankiewicz A et al showed that there was no association between any of the GCR polymorphisms and the need for increased doses of

GCs for asthma.[440]

1.21.4.2 Body mass index and obesity

Young adult male carriers of alleles inducing mild to moderate cortisol resistance are reportedly on average taller and have greater lean mass, while female carriers have a tendency to exhibit lower fat masses, and smaller waist and hip circumferences.[455] It appears that this clear sexual dimorphism seen in the aforementioned study may be modulated by sex steroids. Age may also influence the phenotype. For example in the study, BclI sensitising polymorphism an elevated BMI among middle aged subjects was induced[456] whereas an elderly population may demonstrate a lower BMI[436] which to some degree underpins the unpredictability of the net effect of the GCR polymorphisms.

Recent work has shown that the *Bcl*I (G allele) polymorphism is associated with BMI, conferring an effect size over four successive visits of 1.4 kg/m² and 2.0 kg/m² for heterozygotes and homozygotes respectively, as well as increased fat mass in boys, not preselected for leanness, aged between 8 years and 14 years either pre- or peri-pubertal. These findings were not reproducible in a similar group aged between 13 years and 36 years of age who were leaner, but not preselected for their leanness.[432] The *Bcl*I (G allele) polymorphism is also thought to influence the prevalence of metabolic derangements, with heterozygotes having a greater BMI than wild-type homozygotes. However, Di Blasio et al identified that an increase in BMI in this study population was associated with the combination of both *Bcl*I (G allele) and N363S carriers.[452] Nevertheless, there are several studies that fail to demonstrate an association between the *Bcl*I (G allele) polymorphism and obesity.[457] [458] The presence of the *Bcl*I (G allele) and overfeeding, studied in 12 pairs of identical twin men has been associated with increased body weight, LDLC and abdominal visceral fat.[459] In another study, abdominal visceral fat increased slightly in obese women and men in the presence of the G allele of the *Bcl*I polymorphism, over a 5 year follow-up period.[460] In another study,

the *Bcl*I (G allele) has been associated with an increased cortisol response to a standard meal and visceral obesity, but not generalised obesity, in another study. Individuals harbouring the *Bcl*I (G allele) polymorphism, demonstrated a higher systolic blood pressure and increased visceral obesity, compared with non-carriers of this polymorphism.[461]

Overall, the data available for the *Bcl*I polymorphism, and its link with BMI and obesity are inconsistent, implying that this polymorphism may only exert a small influence or the control of BMI and obesity is subject to multiple and complex pathways.

Lower median mass gain of 2 kg and consequently lower BMI during pregnancy, has been reported among Hungarian women with the ER22/23EK (GAA AAG allele) polymorphism, compared to non-carriers.[462] There was no difference between the BMI of elderly subjects harbouring ER22/23EK polymorphism compared to the wild-type.[458] van Rossum et al showed that some male carriers of the ER22/23EK (GAA AAG allele) polymorphism had greater longitudinal height, equivalent BMI and fat mass, but the lean body mass was increased in male carriers, compared to the wild-type.[455] The ER22/23EK polymorphism did not appear to be universally protective against an elevated BMI or obesity.

It could be expected, that since the N363S (G allele) enhances GC sensitivity that it may be associated with an increased BMI, but available data have been inconsistent. In 375 European subjects examined for the presence of the N363S (G allele), the overall prevalence was 3%, and it conferred a greater waist-to-hip ratio in men, but not in women.[434] Among Australians, the N363S (G allele) polymorphism was shown to confer an elevated BMI.[444] In a heterogeneous population of South Asians, the prevalence of the N363S (G allele polymorphism) was only 0.3% in patients with the metabolic syndrome, indicating that it is a poor marker of obesity.[446] A meta-analysis failed to yield convincing results that the N363S polymorphism (G allele) is associated with obesity in that the BMI was

higher in the population carrying the N363S (G allele) polymorphism when the BMI was less than 27 kg/m2. This finding was not replicated when combining data from both the Spanish and German cohort studies.[460] Rosmond et al also reported no association with either BMI or sensitivity to GCs among 284 Swedish men with the N363S (G allele) polymorphism.[463] In middle-aged males with juvenile onset obesity the N363S (G allele) was counter-intuitively associated with lower fat mass at a given BMI, as measured by DEXA scan.[464] The studies have shown an inconsistent relationship between the N363S (G allele) polymorphism and the development of obesity, suggesting that this polymorphism too has a minor impact on obesity or that the development of obesity is highly complex.

Similarly, Echwald in 2001, showed no association between BMI, waist-hip-ratio or weight gain among 741 obese Danish men and 854 non-obese controls with the N363S (G allele) polymorphism.[465] Another negative study by Halsall et al showed no association between BMI and the N363S (G allele) polymorphism. In 56 obese and 43 non-obese pre-menopausal women, hyperinsulinaemia in obese homozygotes was found, but hyperinsulinaemia was not evident among non-obese homozygous GG carriers of the N363S polymorphism.[433] Individuals with the N363S (G allele) variant conferring increased GC sensitivity, have been shown to be more likely to develop obesity and CVD, compared to their homozygous wild-type carrier counterparts.[434 443 466]

1.21.4.3 Dysglycaemia

Among homozygote carriers with the *Bcl*I (G allele), a significant increase in BMI, abdominal obesity, fasting glucose insulin and homeostasis model assessment-insulin resistance (HOMA-IR) was identified, over a 5-year follow-up period.[460] Weaver et al showed a significant association between homozygous obese carriers of the *Bcl*I (G allele) polymorphism and hyperinsulinaemia.[459] In a group of Swedish men born in 1944 who were homozygous for the *Bcl*I (G allele) polymorphism, a significant association with fasting insulin, fasting glucose and

HOMA-IR was identified.[460]

One prospective study that involved children from the age of 13 years, showed that carriers of the ER22/23EK (GAA AAG allele) polymorphism have a lower tendency to develop impaired glucose tolerance or T2DM.[433 455] Among elderly subjects over the age of 85 years of age, the presence of the ER22/23EK (GAA AAG allele) polymorphism was counter-intuitively associated with higher levels of glycosylated haemoglobin A1C (HbA_1C), considering that this polymorphism ostensibly reduces sensitivity to GCs. The explanation suggested for this unexpected finding was that the GCR polymorphisms may have an altered response in elderly subjects.[458] Pre-term birth is associated with an increased risk of developing the metabolic syndrome. However, among adults who were born prematurely, the ER22/23EK (GAA AAG allele) polymorphism has been found to decrease the risk of developing the metabolic syndrome, possibly due to its reduction of insulin and lower HOMA-IR.[455] Moreover, in severely pre-term subject carriers of the ER22/23EK variant, who were followed up from birth until the age of 19 years of age, appeared to be partially protected against developing the metabolic syndrome, by virtue of a lower HOMA-IR, lower fasting insulin and taller stature, compared to non-carriers of this polymorphism.[467]

The glucose tolerance status in British subjects of European origin was not affected by the N363S (G allele) polymorphism[434] yet in individuals who were administered dexamethasone. this polymorphism was associated with greater suppression of cortisol, but higher insulin levels.[443]

1.21.4.4 Lipids and cardiovascular risk

TThe absolute changes conferred by GCR polymorphisms on lipid and lipoprotein parameters are shown in Table 8. Investigation of GCR polymorphisms in patients with familial hypercholesterolaemia (a sub-group of patients at significantly increased risk of CVD), showed that men carrying the *Bcl*I (G allele) polymorphism

had a 34% increased risk for CVD, compared to the remaining cohort who were negative for the *Bcl*I (C allele) polymorphism. However, this was not related to the severity of the lipid abnormalities after being extensively adjusted for conventional CVD risk factors, suggesting that mortality in this sub-group could be increased through some novel risk mechanism.[420] Homozygotes for the G allele of the *Bcl*I polymorphism had a greater intimal medial thickness of the left common carotid artery (examined for the purposes of consistency), compared to patients who carried at least one C allele.[468] A study of elderly Dutch subjects over the age of 85 years, failed to demonstrate a relationship between the presence of the BclI (G allele) and any of the lipid parameters studied.[458]

Although the ER22/23EK polymorphism ostensibly results in a reduction of GC sensitivity, the clinical findings of a number studies have inconsistently demonstrated this and consequently have shown a lack of metabolic protection in the presence of this polymorphism. Reduced fasting insulin and lower TC and LDLC have been reported in patients harbouring the ER22/23EK (GAA AAG allele) polymorphism.[450] The ER22/23EK (GAA AAG allele) polymorphism has also been recorded as being associated with reduced hs-CRP and improved survival rates in elderly men.[435] Koeijvoets et al found no association between the ER22/23EK (GAA AAG allele) polymorphism and CVD risk in patients with familial hypercholesterolaemia.[469] The ER22/23EK (GAA AAG allele) polymorphism has been associated with reduced GC sensitivity and subjects with this variant, demonstrate lower fasting insulin, TC and LDLC.[450]

The N363S (G allele) polymorphism was not associated with elevated blood pressure, TC, TG, LDL or HDLC in British subjects of European origin.[434] In a study of Caucasians older than 85 years of age in the Netherlands, the N363S (G allele) polymorphism was associated with higher concentrations of LDLC and TG, compared to non-carriers.[458] Dobson et al, showed no association between the N363S (G allele) polymorphism and serum lipids, but this polymorphism was

associated with increased waist-to-hip ratio in male Caucasians.[434] Lin in 2003 showed that the N363S (G allele) polymorphism was associated with coronary artery disease (categorised as unstable angina, stable angina or no angina), elevated TC, TG and TC/HDL ratios. Interestingly, the risk for coronary artery disease was associated with the N363S (G allele) and was influenced by body mass, that is lean patients with CVD exhibited a three-fold higher frequency and obese patients had a five-fold higher frequency of harbouring this polymorphism, compared to non-carriers. This suggests that this genetic influence on coronary artery disease may be mediated to some degree by mass.[470] Thus certain polymorphisms within the GCR gene that promote GC sensitivity may contribute to degrees of obesity, body fat distribution and enhanced CVD risk.[418] The study by Di Blasio et al reveals that two sensitising GCR polymorphisms may act synergistically, such that the net effect is potentiated. The *Bcl*I (G allele) polymorphism in combination with the N363S (G allele) showed significantly higher TC and LDLC, compared to the N363 carriers alone.[452] Table 8 summarises some of the findings in the literature on the impact of three selected GCR polymorphisms on lipids, lipoproteinis and BMI.

Table 8: Summary of the absolute lipid, lipoprotein and BMI differences (conferred by the presence of three glucocorticoid receptor polymorphisms

Polymorphism	Lipid fraction/ BMI	Absolute change	Reference
ER22/23EK (rs6189) (GAA AAG allele)	TC	-0.74 mmol/L	Van Rossum EF Diabetes 2002 [450]
ER22/23EK (rs6189) (GAA AAG allele)	LDL	-0.8 mmol/L	Van Rossum EF Diabetes 2002 [450]
N363S (rs6195) (G allele)	LDL	+0.56 mmol/L	Kuningas M, Biogerontology 2006 [458]
N363S (rs6195) (G allele)	TG	+0.21 mmol/L	Kuningas M, Biogerontology 2006 [458]
N363S (rs6195) (G allele) **plus *Bcl*I (rs41423247)** (G allele) carriers versus **N363S (rs6195)** (G allele) alone	TC	+1.63 mmol/L	Di Blasio A, Clinical Endocrinology 2003 [452]
N363S (rs6195) (G allele) **plus *Bcl*I (rs41423247)** (G allele) carriers versus **N363S** (G allele) alone	LDL	+1.60 mmol/L	Di Blasio A, Clinical Endocrinology 2003 [452]
N363S (rs6195) (G allele)	TC	+0.3 mmol/L	Lin RC, Hypertension 2003 [466]
N363S (rs6195) (G allele)	TG	+0.4mmol/L	Lin RC, Hypertension 2003 [466]
***Bcl*I (rs41423247)** (G allele)	BMI	+1.4 kg/m^2 (heterozygotes) +2.0 kg/m^2 homozygotes)	Voorhoeve P, Clinical Endocrinology 2009 [432]
ER22/23EK (rs6189) (GAA AAG allele) versus non-carriers	BMI (during pregnancy)	+0.9 kg/m^2	Bertalan R, Gynaecological Endocrinology 2009 [462]

Polymorphism	Lipid fraction/ BMI	Absolute change	Reference
N363S (rs6195) (G allele)	BMI	+1.9-4.3 kg/m^2 (heterozygotes) +2.5-12.4 kg/m^2 (homozygotes	Lin RC, British Medical Journal 1999 [444]
N363S (rs6195) (G allele)	BMI	+0.18 kg/m^2	Marti A, BMC Medical Genetics, 2006 [471]

TC: total cholesterol

LDL: low density lipoprotein

TG: triglycerides

+: absolute increase

-: absolute reduction

A,C,G = DNA bases

BMI: body mass index

References provided in superscript

1.21.4.5 Reduced bone mineral density

Bone mineral density was reduced among patients with Cushing's syndrome in two femoral regions among the carriers of the *Bcl*I (G allele) homozygous form, compared to the wild-type, suggesting that the complications that may result from endogenous hypercortisolism are likely to be modified by the presence of GCR polymorphisms.[472] In the Longitudinal Ageing Study Amsterdam (LASA), the serum fasting cortisol was associated with reduced bone mineral density at the femoral neck; in particular, female homozygous *Bcl*I (G allele) polymorphism carriers had the highest serum fasting cortisol, but the lowest bone mineral density at the lesser trochanter. These findings were not replicated in the male sub-group.[473] As far as is known, there are no known data on the relationship between the remaining GCR polymorphisms and reduced bone mineral density.

1.21.4.6 Longevity

None of the three aforementioned polymorphisms of the GCR in participants older than 85 years of age (Leiden 85-plus study), was associated with either CV or all-cause mortality.[458] On the other hand, children with acute lymphoblastic leukaemia, treated with antineoplastic agents including steroids and homozygous for the *Bcl*I (G allele) polymorphism, had reduced survival rates.[474] van Rossum et al suggested that the ER22/23EK (GAA AAG) polymorphism may confer survival benefit following the observation that the prevalence of this polymorphism was highest in the oldest of an elderly population from the Netherlands, after 4 years of follow-up. Also of interest is that the carriers of the ER22/23EK (GAA AAG allele) polymorphism had 50% lower CRP levels.[475]

1.21.4.7 Psychiatric and psychological manifestations

The cortisol responses to psychological disorders or challenges in relation to the GCR polymorphisms are unpredictable and appear also to be affected by gender. Wust et al showed that the salivary cortisol response was highest in carriers of the N363S (G allele) polymorphism, compared with the wild type (A allele) and the *Bcl*I (G allele) carriers had by contrast, a diminished response following exposure to a psychosocial stress.[476] Kumsta et al demonstrated highest cortisol responses among women with the *Bcl*I (homozygous for the G allele) polymorphism to the Trier Social Stress Test (TSST), which is an experimental procedure used to induce stress under laboratory conditions. By contrast, *Bcl*I (G allele) male homozygotes subjected to the same stress had the lowest salivary cortisol response when compared to their respective wild-types.[477] Lower cortisol levels were found in Vietnam war veterans with post-traumatic stress disorder, harbouring the *Bcl*I (G allele) polymorphism.[478]

Depression is biologically linked to excess levels of cortisol, and yet paradoxically in a cohort of subjects with major depression, the ER22/23EK (GAA AAG allele) polymorphism was more prevalent than in healthy control subjects. van Rossum

et al reported that ER22/23EK (GAA AAG allele) polymorphism was associated with recurrent major depression and a more rapid response to antidepressant therapy.[479]

There are isolated studies examining the relationships between salivary cortisol and GCR polymorphisms. In a study by Wust et al the interaction between psychological stress and GCR polymorphisms revealed that N363S (G allele) carriers had the greatest cortisol response as measured by salivary cortisol. In a study comparing the salivary cortisol exposure response following a stressor, N363S (G allele) carriers had the greatest cortisol exposure taken at 2 min, 15 min, 25 min, 45 min, 60 min, 75 min and 105 min after a TSST.476 Among 601 healthy subjects subjected to the TSST, male carriers of the N363S (G allele) polymorphism exhibited the highest salivary cortisol response, but no increase was observed in women participating in this study.[477] Repeated noxious stimuli may induce habituation and in those carriers of the N363S (G allele) polymorphism, a weak trend towards lower excursions of salivary cortisol was observed, compared to the remaining genotypes.[480]

1.21.4.8 Autoimmune disease

Carriers of the *Bcl*I (G allele) polymorphism appeared to have a less advanced stage of Graves' ophthalmopathy, which may be explained by the likely enhanced sensitivity to endogenous GCs, with partial dampening of the immune response.[483] There are conflicting reports demonstrating a relationship with inflammatory bowel disease and these GCR polymorphisms. For example, Crohn's disease demonstrated a greater frequency of subjects with *Bcl*I (G allele) polymorphism in one report, whereas another report showed no relationship.[435] Further studies are required to resolve the precise relationship between this polymorphism and the development of inflammatory bowel disease. The *Bcl*I (G allele) polymorphism was not associated with rheumatoid arthritis, among Korean patients.[411]

The ER22/23EK (GAA AAG allele) polymorphism was associated with a far more aggressive course of multiple sclerosis in contrast to no identifiable association with other autoimmune diseases.[481-483] No association was identified between the ER22/23EK polymorphism and the risk for developing myasthenia gravis.[484]

The N363S (G allele) polymorphism in several studies did not appear to influence the risk of rheumatoid arthritis, whereas one Dutch study suggested a lower risk.[434] Overall, the association between autoimmunity as a sub-group of disorders and GCR polymorphisms is at best tenuous.

1.21.4.9 Miscellaneous

As elevated levels of cortisol have been implicated in the pathogenesis of dementia, the Rotterdam study[442] found that the ER22/23EK (GAA AAG allele) polymorphism was associated with a reduced incidence, implicating a possible role of this polymorphism in protecting against dementia. The ER22/23EK (GAA AAG allele) polymorphism modified the relationships between serum cortisol and grip strength, but not after stratifying for the individual polymorphisms, possibly due to the small number of subjects.[485]

Decreased inhibition of interleukin-2 (IL-2) production is expected in the presence of GCs, thus homozygote carriers of the N363S (G allele) demonstrated higher concentrations of this cytokine.[439] Peeters et al reported that higher salivary cortisol was associated with reduced grip strength, the carriers of the N363S (G allele) polymorphism in the middle serum cortisol quartiles had the highest grip strength.[485]

1.21.4.10 Hypothesis of GCR polymorphisms in Addison's disease

There is a dearth of information on whether GCR polymorphisms play a role in the development of GC-related side-effects in individuals receiving hydrocortisone replacement for Addison's disease. In addition, there is uncertainty, as to whether the empiric dose of hydrocortisone needs to be altered in the presence of certain

GCR polymorphisms.

1.22 Conclusions

Major advances in our understanding of the embryological development of the adrenal glands have occurred as a result of molecular biological techniques. This has provided insight into the various causes of congenital adrenal gland hypoplasia.

Addison's disease occurs in 93-144 people per million people in the Western world, where autoimmunity is the most common underlying aetiology. This is in contrast to earlier studies that have attributed tuberculosis and idiopathic causes to be the most abundant underlying aetiologies. It might be expected that certain HLA DQB1 alleles could be associated with autoimmune Addison's disease, in which a wide array of autoantibodies is frequently seen due to disruption of global autoantibody production. As there is a high prevalence of HIV in South Africa, specific adrenal infections with subsequent hypoadrenalism could occur more commonly than in other parts of the world where HIV is less prevalent and autoimmunity may be a less common cause of Addison's disease.

GCs may modulate multiple pathways governing lipid and lipoprotein metabolism. It is thus expected that Addison's patients could exhibit alterations in lipid and lipoprotein metabolism. If sufficiently adverse lipid profiles are identified in this sub-group of patients, it could explain the more than double risk of CVD and premature mortality.

There are multiple methods to monitor hydrocortisone replacement, none of which is ideal. Since salivary cortisol is easily accessible, and has been found to correlate with plasma cortisol, it meets the criteria for monitoring hydrocortisone replacement therapy in patients in the comfort of their own homes. Although

GCR polymorphisms may modulate the effects of circulating cortisol, and have been associated with metabolic abnormalities, the absolute changes are small. It is anticipated, that should metabolic differences be identified in association with these GCR polymorphisms, these are likely to be small.

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Chapter 2
Rationale, aims and objectives

2.1 Rationale

My long-standing interest in Addison's disease was kindled by the awareness that this rare disease is life-threatening if not diagnosed and managed appropriately, and that there is a dearth of knowledge pertaining to Addison's disease in South Africa.

In the early stages of my odyssey into investigating Addison's disease, I read several reports of cancer autopsies that confirmed the high prevalence of adrenal metastases, yet few verified the presence of primary hypoadrenalism using conventional diagnostic criteria. I addressed this unresolved question by determining the prevalence of primary hypoadrenalism, using validated diagnostic criteria in 30 patients not pre-selected for adrenal metastases with advanced (stage III or IV) bronchogenic carcinoma. Two patients had definitive evidence for adrenal insufficiency, representing a 6.7% (95% confidence interval 0.8 - 22.1%) prevalence of adrenal insufficiency,[1] which is substantially lower than the 33% reported by Redman et al.[2] This study prompted the work covered by this thesis, since I was concerned about the possibility that primary hypoadrenalism, which is a highly treatable condition, could be under-diagnosed, inadequately managed and its finer nuances, overlooked.

Despite the large body of evidence, that autoimmunity is the predominant cause for Addison's disease in the developed world, uncertainty exists as to whether it is also the case in South Africa.[3-6] This developing country is characterised by a heterogeneous population, lack of uniform access to healthcare, and multiple disease burdens, including epidemics of tuberculosis and HIV, both of which are well-established causes for Addison's disease.[7 8]

Given the heterogeneous ethnic background of South Africans, it is also unclear whether patients with autoimmune Addison's disease share the same HLA DQB1 alleles described from the Western world. It was considered that a large multicentre cohort study could be instrumental in determining both the underlying aetiology of Addison's disease in South Africa and the HLA DQB1 genotypes.

Until recently, it was believed that patients with Addison's disease had a comparable survival rate to the background population. However, a Swedish study indicated that the risk of death was two-fold higher in treated Addison's disease, due to predominantly CVD and cerebrovascular disease.[9] Although Addison's disease per se, could have contributed to this accelerated mortality, supra-physiological levels of GCs may have resulted in abnormal lipid and lipoprotein metabolism.

Oral hydrocortisone is the most commonly used therapy for GC replacement in Addison's disease. Various methods have been used to monitor this therapy, but none is ideal. Salivary cortisol is easily accessible and can be studied in the comfort of patients' own homes. Several studies in Addison's disease have shown reasonably good correlation between plasma and salivary cortisol.[10 11] Some authors on the other hand, have debated the validity of salivary cortisol monitoring, due to its high variability.[12 13] An area that has not been analysed much is whether patients on usual hydrocortisone replacement therapy have the equivalent cortisol exposure to healthy subjects' endogenous cortisol profiles. If it turns out that patients on usual hydrocortisone replacement are exposed to higher cortisol concentrations than healthy control subjects' endogenous exposure, it may predispose patients to metabolic derangements, which are well-known to occur with supra-physiological doses of GCs.

Replacement therapy in Addison's disease has to a large extent been empirically determined. There has been some appreciation of the potential of GCR polymorphisms to modulate the effect of circulating cortisol levels. Three important

GCR polymorphisms have been identified, two of which exert a sensitising effect and another that confers a degree of resistance to cortisol. It is uncertain as to what extent the presence of the GCR polymorphisms may either modulate empiric doses of hydrocortisone replacement therapy or induce metabolic alterations in the context of Addison's disease.

2.2 Aims

The overall aim of this study was to determine the aetiopathogenesis, CV and metabolic complications, and pharmacogenomics, in a cohort of patients with Addison's disease in South Africa

The primary and secondary objectives of this study were:

1. To examine the aetiopathogenesis of Addison's disease in South Africa and to determine whether specific HLA DQB1 alleles are associated with autoimmunity.[14]
   (i) To determine the underlying aetiology of Addison's disease in South Africa, using special investigations, acquired through collaboration with centres in the USA and Sweden.
   (ii) To determine whether in this South African cohort, certain HLA DQB1 alleles may predispose to autoimmunity.

2. To determine the cause for the reported increased CV mortality in Addison's patients by examining lipids, lipoproteins and markers of CV inflammation.
   (i) To establish whether patients with Addison's disease have adverse lipid profiles, raised markers of inflammation and CVD Framingham risk than controls.

[14] Title: Autoimmunity predominates in a large South African cohort with Addison's disease of mainly European descent, despite long-standing disease and is associated with HLA DQB*0201.
Ian Ross was the principal author who conceived the study, recruited patients, performed data analysis and wrote the manuscript.[14] Co-authors of the paper included a doctoral supervisor, a statistician and overseas individuals, who performed special serum and genetic investigations.

(ii) To determine if a correlation exists between doses of hydrocortisone replacement and the parameters of evaluating lipid and lipoprotein metabolism.

(iii) To compare lipids and lipoproteins in a sub-group of South African Addison's patients matched with a Swedish Addison's sub-group for age, gender, ethnicity and BMI.

3. To test the hypothesis that patients with Addison's disease, who are fully replaced on hydrocortisone and fludrocortisone, may be subject to supra-physiological doses of GCs, as examined using salivary cortisol AUC.

   (i) To determine the salivary cortisol AUC for patients with Addison's disease on full replacement, and compare these to healthy control subjects' endogenous salivary cortisol concentrations.

   (ii) To determine the optimum time of sampling of salivary cortisol in patients and healthy control to most accurately reflect the salivary cortisol peak and the AUC.

   (iii) To determine whether an increased salivary cortisol AUC translates into abnormal lipid, lipoprotein and markers of CV inflammation.

4. To determine whether metabolic alterations are associated with GCR polymorphisms to warrant modifications of hydrocortisone replacement, the data for which have so far been empiric.

   (i) To explore the role of GCR polymorphisms in influencing metabolic parameters among patients with Addison's disease, by comparing or correlating GCR genotypes with clinical parameters including BMI, TC, TG, HDLC, LDLC, hs-CRP, NEFA, small dense LDL, TSH and hydrocortisone dose.

   (ii) To determine whether clinical differences exist among patients who harbour GCR polymorphisms with either increased or decreased sensitivity versus those with wild type.

Chapter 3 details the methods used for the collection of this cohort and describes their clinical and demographic features. Chapter 4 describes the aetiopathogenesis of Addison's disease, as well as genetic factors predisposing individuals to autoimmune Addison's disease in South Africa. Chapter 5 describes the observed lipids, lipoproteins and markers of CVD in patients with primary hypoadrenalism. Chapter 6 introduces the concept of monitoring replacement therapy in Addison's disease by using salivary cortisol day curve measurements. Chapter 7 is dedicated to describing the effect of GCR polymorphisms on the sensitivity to cortisol in patients with Addison's disease. The concluding chapter, Chapter 8, summarises the major findings of these various studies, and provides recommendations that will impact on management of patients with Addison's disease.

It is hoped that this body of work will contribute to raising awareness of this highly treatable condition amongst physicians working in our resource poor environment. If, in addition, it heralds an appreciation for the potential metabolic consequences that can occur with Addison's disease, and lowers the threshold for initiating corrective therapy, it would have contributed somewhat to the outcome of patients with this condition.

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Chapter 3
Methods and description of the cohort

3.1 Background

Several factors peculiar to the health-care system in South Africa influenced recruitment of patients in this study. These are outlined below.

3.1.1 The health-care systems in South Africa

South Africa has a population of more than 47 million people. In the absence of national health insurance, there is unequal access to health-care. The health-care system consists of a vast public sector and a small, but expanding private sector.[1] The latter serves 18% of the population, while the public health sector serves 82% of the population, predominantly the poor working class indigent, and is oversubscribed. The public health sector is challenged by a significant human resource crisis, HIV and tuberculosis epidemics, and is also burdened by non-communicable diseases. Despite the end of apartheid, racial and gender discrimination, income inequalities, and extreme violence continue to impede its function.[2] Health-care varies from the most basic care, offered free of charge by the state, through to highly specialised health services at quaternary level and private sectors for those individuals who are members of a medical insurance company or those who can afford to pay for it. There has been a steady increase in the number of private hospitals; in 2005, there were 161 and in 2010, there were approximately 238, with 142 of these situated in urban areas.[3 4] The mining sector, which provides health-care for its employees, is responsible for a further 60 hospitals around the country. Secondary care hospitals, or district general hospitals, are found in most of the larger towns throughout South Africa.[2] While some secondary care hospitals have computerised data, many do not keep any records, highlighting some of the challenges faced in identifying patients with a particular disorder.[5] Although tertiary hospitals offer specialised medical care, often

in association with a medical school or university, they do not have sub-specialist endocrinology facilities. Similarly, the eight medical schools in South Africa do not universally offer specialised endocrine services. There are perceptions among certain strata of the population that the current public health service offers inferior health-care.[6]

Five of the nine provinces in South Africa have quaternary hospitals (major teaching hospitals with sub-speciality facilities) with medical schools attached. However, four of the provinces do not have specialised endocrine clinics. The quaternary hospitals receive referrals from the neighbouring provinces that do not have quaternary centres.

3.2 Method of registry compilation

Since databases in South Africa were not available for Addison's disease, a systematic approach was adopted of initially inviting patients attending quaternary hospitals, followed by patients attending tertiary hospitals and private health-care facilities. This was followed by inviting prospective participants attending both secondary and primary health-care facilities.

3.2.1 Ethics and informed consent

Approval to conduct the study was obtained from the Research and Ethics Committee of the University of Cape Town. Ethics approval was also obtained from the respective research and ethics committees overseeing the various faculties of health sciences including the Nelson Mandela School of Medicine, University of KwaZulu-Natal, University of Stellenbosch, University of the Free State, University of Pretoria and the University of Witwatersrand. All participants signed written informed consent.

3.2.2 Patient enrolment

The outline of the procedure followed in order to identify cases of Addison's disease is shown in Figure 11. As Addison's disease is likely to be diagnosed and managed by endocrinologists rather than generalists, the first phase was to contact all quaternary hospitals to compile a registry of Addison's patients.

Patients with Addison's disease who attended the endocrine clinic at Groote Schuur hospital, which is affiliated to the University of Cape Town, were sequentially invited by the medical staff at their routine clinical appointments to participate in the study, as there were no databases to indicate their personal and clinical details.

In the next phase, all other endocrinology divisions attached to quaternary hospitals (Pretoria Academic hospital, Albert Luthuli hospital, Universitas hospital, Pelonomi hospital, Johannesburg General hospital, Chris Hani Baragwanath hospital and Tygerberg hospital) were contacted to invite their patients with Addison's disease to enrol in this registry. In the subsequent phase, tertiary hospitals without endocrinology divisions were then also contacted to invite patients with Addison's disease to participate in this study. These hospitals included Helen Joseph, Livingstone, Garankuwa, Paarl, George, Cecilia Makiwane and Nelson Mandela Academic hospitals. All private endocrinologists were contacted to enhance the referral base, using the society of endocrinology membership {Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA)} database. All specialist physicians (internists) in the Western Cape were accessed using telephone directories, issued by the postal services, and letters were written to each of them.

A private commercial database (MedPages) of medical specialists and general practitioners sent 9 600 personalised e-mails to all specialist physicians, paediatricians and general practitioners registered with this organisation.

Specialist physicians or paediatricians were thought more likely to be involved in the management of Addison's disease, compared with other disciplines.

As Addison's disease is designated as a medical condition that enjoys the prescribed minimum benefit, it is a statutory requirement in South Africa that patients belonging to a medical aid have the total cost of their treatment reimbursed.[7] Although the medical aid or medical insurance companies were able to access the names of the patients suffering from Addison's disease, only the names of their treating physicians were communicated to the researcher, as divulging the names to a third party would have represented a breach of confidentiality. Thereafter, the treating physician was requested to invite his or her patients to participate in this study.

A facility database of both private and public health-care was constructed, which included all primary, secondary, tertiary and quaternary health-care in South Africa. This was created using the Internet and the following key words were entered into the search engine for each of the nine provinces: "hospitals", "clinics", "day hospitals", "community healthcare centres", "district hospitals", "district general hospitals", "secondary hospitals", "private hospitals", "Department of Health" "list of hospitals", "primary care facilities", "secondary care facilities", "secondary tier facilities", "tertiary care facilities", "quaternary care facilities", "rural doctors association", "netcare", "medical centre", "lifecare", "intercare", "melomed", "medi-clinic", "academic hospitals" "private doctors", "Hospital Association of South Africa" and "private medical practitioners".

From each of the websites, the following information was extracted where possible: the name of the hospital facility, the address and telephone number, the name of the superintendent or chief executive officer, the fax number and the name of the liaison officer. Medical practitioners, or in the case of facilities without doctors, nurses and nurse practitioners were asked to identify patients with

Addison's disease. Letters were written to superintendents of hospitals, medical practitioners and specialists, asking them to identify patients with Addison's disease. They were also asked to inform their staff of the Addison's disease national registry. The diagnosis of Addison's disease was made on the basis of a suggestive clinical presentation, low basal cortisol level and simultaneously elevated ACTH concentration, or where indicated, a peak cortisol, following 250 µg ACTH stimulation, of less than 550 nmol/L associated with a basal raised plasma ACTH, exceeding 10.1 pmol/L. These criteria have been published previously.[8]

Although patients may have been recruited through their general practitioners, Addison's disease was confirmed in each case by a specialist internist, paediatrician or endocrinologist. They were also only considered eligible if they were resident in South Africa.

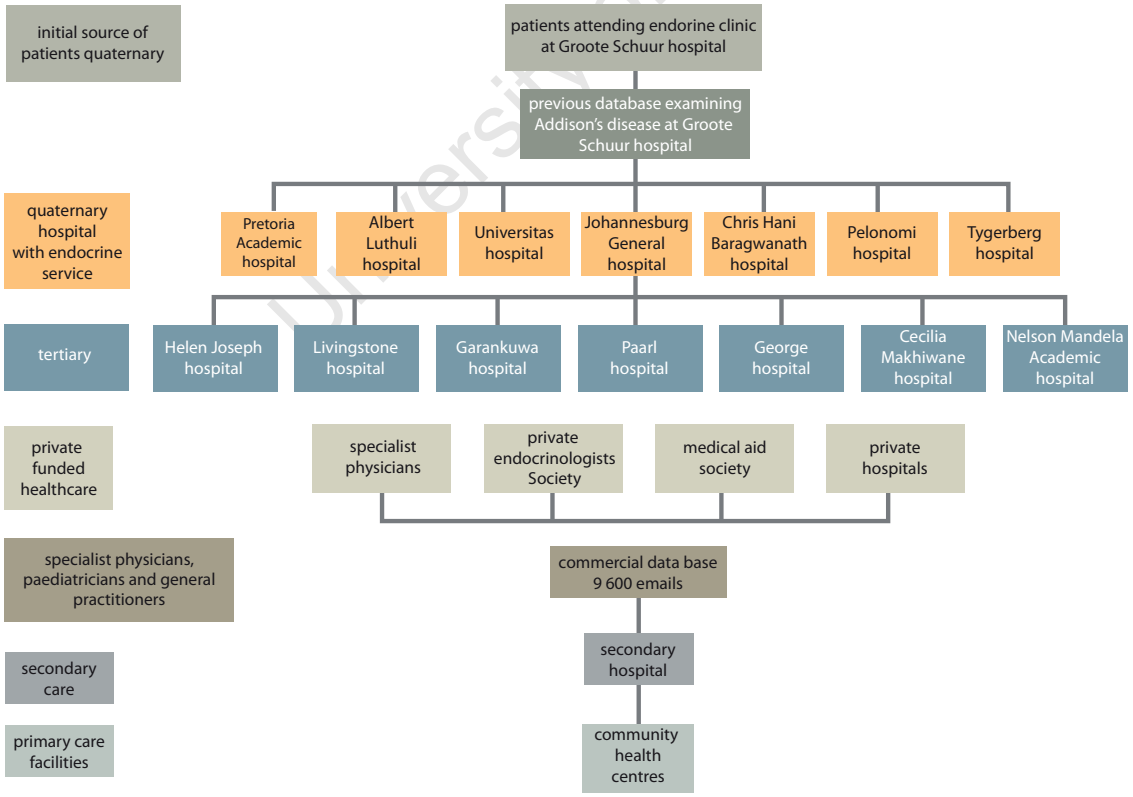

Figure 11: Systematic outline of the procedure followed for identifying patients with Addison's disease

3.3 Pattern of referrals

Overall, the response rate was low, but this was expected in view of the rarity of this disease. As seen in Figure 12, the majority of cases (46.2%) were from tertiary referral centres, followed by quaternary (44.2%), secondary (6%) and primary level sources (3.4%).

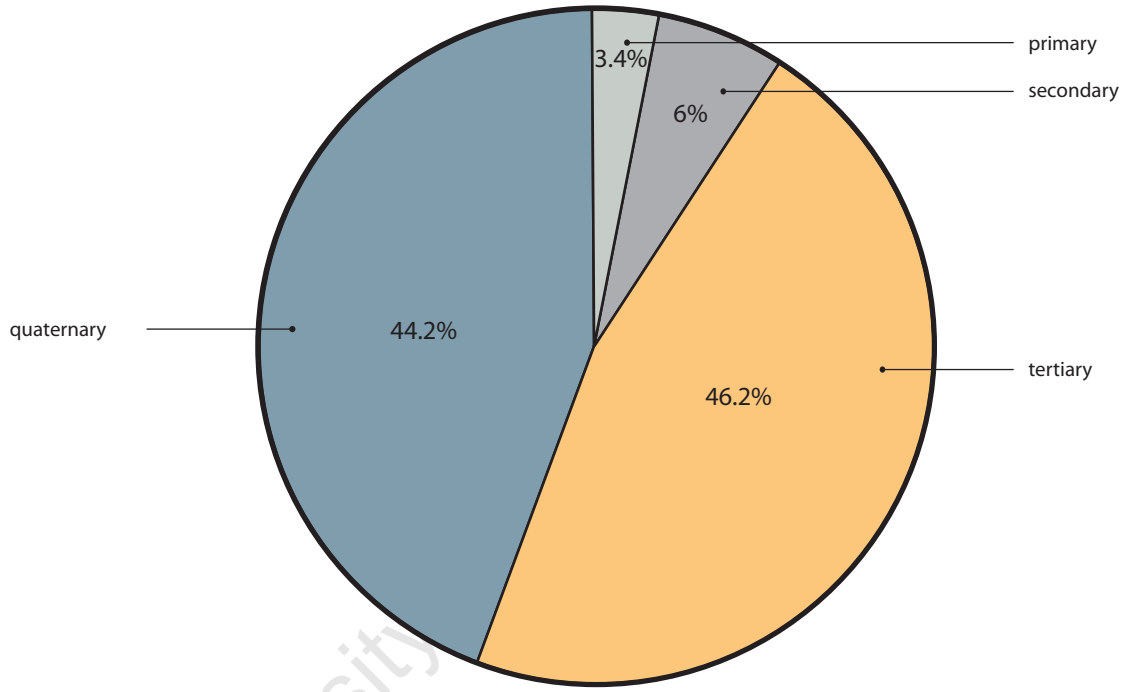

Figure 12: Proportion of referrals from differing levels of health-care in South Africa primary (3.4%), secondary (6%), tertiary (46.2%), quaternary (44.2%)

3.4 Data captured in the registry

The following data were entered onto a spreadsheet: age at enrolment, date of birth, gender, ethnicity, duration of symptoms prior to diagnosis and the symptoms at presentation, duration of Addison's disease, time elapsed between diagnosis and onset of first symptoms, co-morbidity, associated autoimmune conditions, family history of autoimmune disease and treatment. Since European or North American Addison's patients were most likely to have underlying autoimmune Addison's disease, patients in this cohort were evaluated for the presence of positive foreign ancestry as defined by either a first or second degree foreign

relative who was born in either USA or Europe. The presence of primary hypothyroidism, and in particular, the approximate time of its onset was noted.

The total daily doses of fludrocortisone and hydrocortisone replacement therapy were included in the database. The hydrocortisone dose was corrected for weight and body surface area. All chronic medication use and risk factors for CVD were noted, including smoking or tobacco exposure, history of lipid abnormalities, hypertension and diabetes.

3.4.1 Anthropometric data

Height and mass were obtained where possible, which allowed the body BMI and body surface area to be calculated for each patient and control subject. One blood pressure measurement was recorded for each patient, where possible.

3.4.2 Biochemical data included in the registry

The biochemical data were obtained by reviewing medical notes, obtaining transcripts of the source data and by examining archived results in the relevant pathology laboratories. The following biochemistry results at diagnosis of Addison's disease were entered into the database where available: serum sodium, potassium, basal and stimulated plasma cortisol, basal plasma ACTH, serum aldosterone, plasma renin, thyroid stimulating hormone (TSH), thyroxine (T4), and hydroxycobalamin (Vitamin B12).

3.4.3 Investigations performed at enrolment

A number of investigations were performed at the time of patient enrolment. Their detailed methods are described in subsequent chapters. Autoantibodies, biochemical markers of CV risk, HLA DQB1 alleles and genotypes, salivary cortisol, serum TSH, and VLCFAs were investigated.

3.4.3.1 Autoantibodies

ACA and 21-hydroxylase autoantibodies were assayed to confirm autoimmune primary adrenal gland failure. Other clinical autoimmune conditions known to coexist with Addison's disease were screened using appropriate autoantibody tests including tissue transglutaminase, antithyroperoxidase, antithyroglobulin, parietal cell, ovarian, placental, testicular autoantibodies, islet cell and anti-GAD65. This panel of autoantibodies, excluding 21-hydroxylase and tissue transglutaminase autoantibodies was kindly measured by Professor William Winter's laboratory, Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville USA. Both 21-hydroxylase and tissue transglutaminase autoantibodies were kindly measured by Professor Anders Karlsson at the Department of Clinical Immunology, University hospital, Uppsala, Sweden.

3.4.3.2 Biochemical markers of cardiovascular risk

TC, TG and HDLC were measured, but LDLC was calculated using the Friedewald equation. In addition, LDL particle size was measured to determine additional CV risk in both the patients and controls. NEFA and random blood glucose (RBG) were performed in Professor David Marais' laboratory at the University of Cape Town. Professor Gudmundur Johannsson kindly performed the hs-CRP levels in Gothenburg, Sweden.

3.4.3.3 Genetic analyses

The HLA DQB1 alleles and genotypes were recorded for each of the patients and controls. These were kindly performed by Dr She, Centre for Biotechnology and Genomic Medicine, Medical College of Georgia, USA. Screening for GCR polymorphisms took place in the inherited metabolic disease laboratory at the University of Cape Town.

3.4.3.4 Salivary cortisol analyses

This analysis was performed by the National Health Laboratory Service (NHLS) in Cape Town.

3.4.3.5 Serum thyroid stimulating hormone (TSH)

Serum TSH was assayed by a private, accredited pathology laboratory, Davies and partners in Cape Town.

3.4.3.6 Very long chain fatty acids (VLCFAs)

Screening for elevated VLCFA concentration was kindly performed by Professor Japie Mienie from the School of Biochemistry, Northwest University Potchefstroom, South Africa.

3.5 Healthy control recruitment

Healthy control subjects were enrolled in the blood donor clinic of the Western Cape. Each control subject was matched as far as was possible to a patient in terms of age, gender, BMI and ethnicity.

3.6 Description of the cohort

There were 161 patients who were referred for enrolment in the South African Addison's study. Seven patients with an original label of Addison's disease were excluded: two had a normal ACTH stimulation (Synacthen®) test, two had secondary hypoadrenalism, one had a bilateral adrenalectomy for Cushing's disease, and two had suppression of the HPA axis, related to previous steroid use for another indication. Three patients declined to participate, citing personal reasons and a further three patients were too late to be enrolled in this observational study. Thus, there were a total of 148 patients who were enrolled in this study (Figure 13).

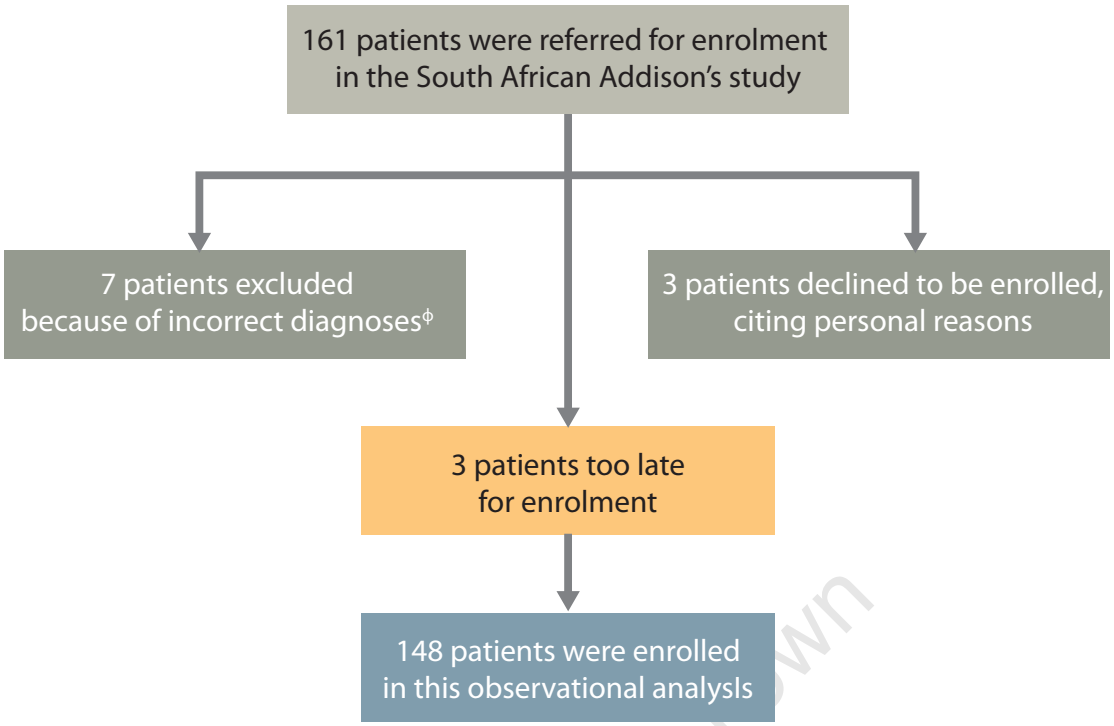

Figure 13: Pattern of enrolment for this observational analysis.

[Φ]Seven patients were excluded as two had a normal ACTH stimulation test, two had secondary hypoadrenalism, one had a bilateral adrenalectomy for Cushing›s disease, and two had suppression of the hypothalamic-pituitary adrenal (HPA) axis, related to previous steroid use for another indication.

3.6.1 Demographic Data

The majority of the cohort was women (62%). The median and interquartile age range (IQR) of patients at enrolment was 46.0 (32.0-61.0) years, with a wide range from 2.8-88.0 years. The median and IQR age at initial diagnosis of Addison's disease however, was 34.0 (20.0-45.0) years (range 0.02-77.0 years), indicating that at the time of enrolment, the patients, on average, had been diagnosed with Addison's disease 12 years previously or longer. The majority of the cohort was diagnosed with Addison's disease at an age of age >20 years (Figure 14). White patients (66%) contributed to the majority of the cohort, followed by mixed ancestry participants (23%). There were few Asian (3%) and black participants (8%) in the cohort (Figure 15). This is in contrast to the background population in which 79.2% of the population are black, 9.2% are white, 9.0% are mixed ancestry and

2.6% are Asian.[9] Of these, 34% confirmed that either their first or second degree relatives were of foreign ancestry, according to the predetermined definition. The majority of patients lived in urban areas (87%) as shown in Figure 16, particularly Cape Town, Johannesburg and Durban, where most of the specialised clinics for endocrinology are situated. Isolated pockets of patients were identified in the northern part of the Western province, southern Cape and various districts of KwaZulu-Natal. Looking at the ethnic specific prevalence among white patients, the prevalence is 20-25 per million which is still lower than that recorded in western countries.

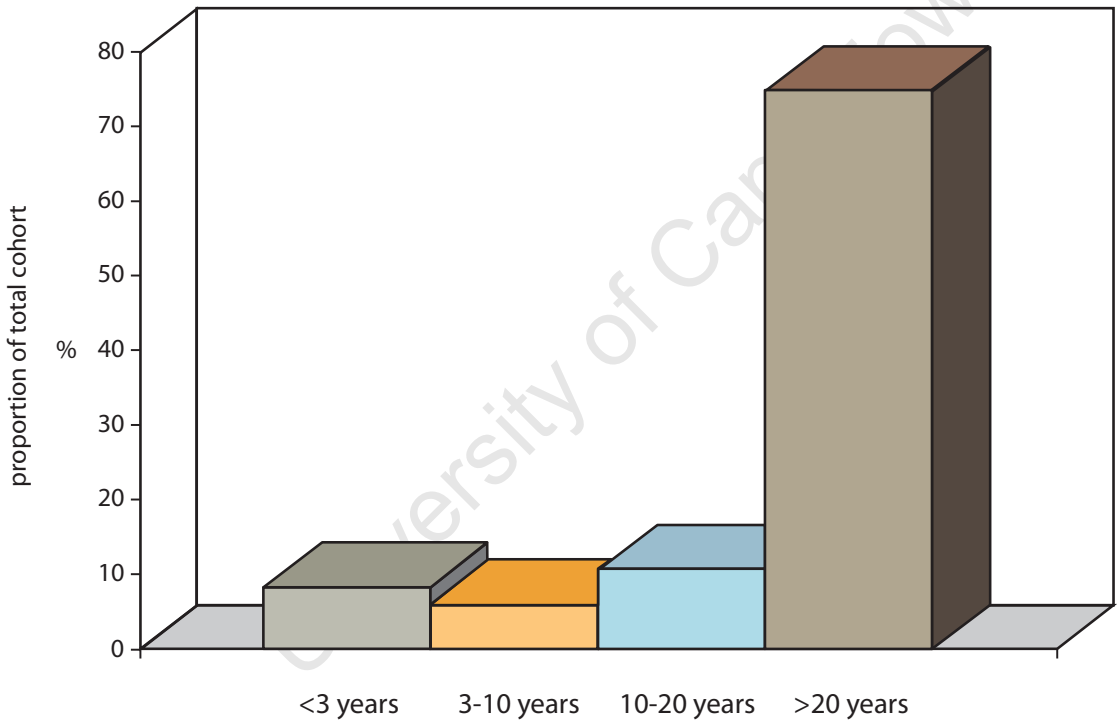

Figure 14: The proportion of the South African Addison's disease patients diagnosed at various chronological ages

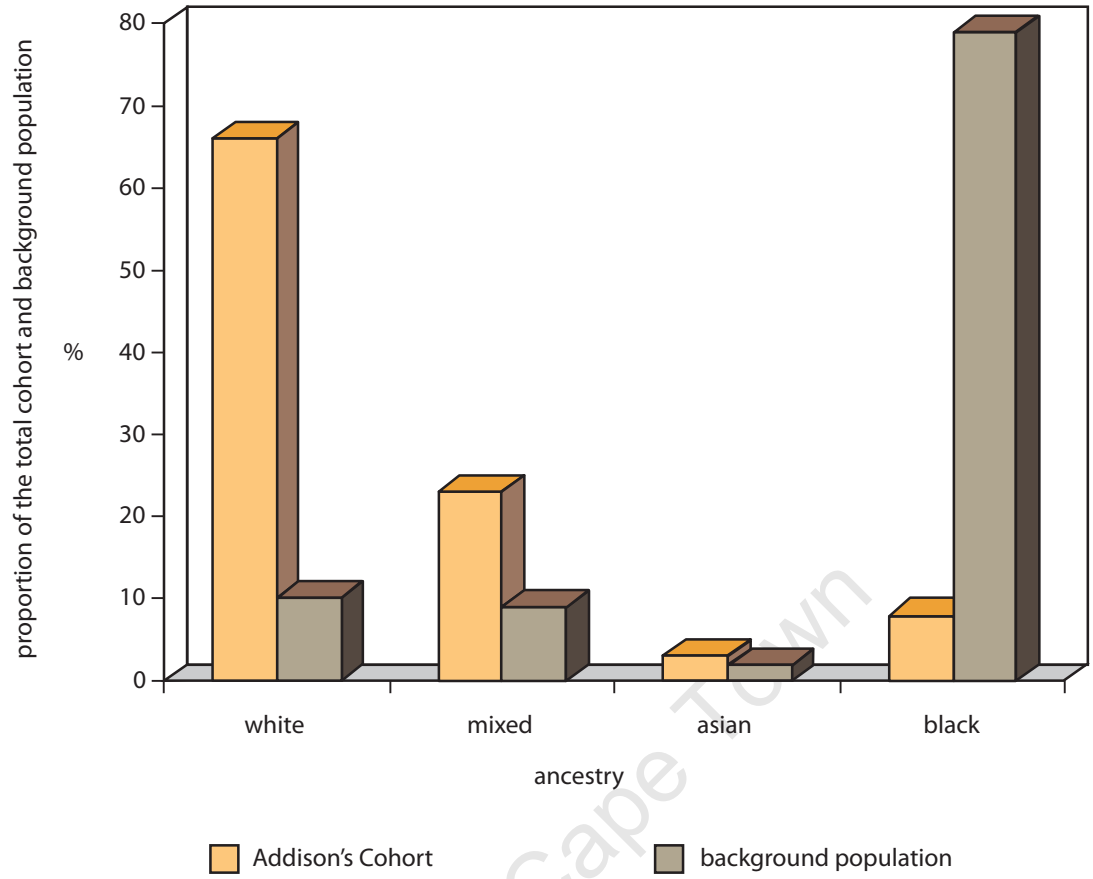

Figure 15: Ethnic distribution of the cohort and the background South African population

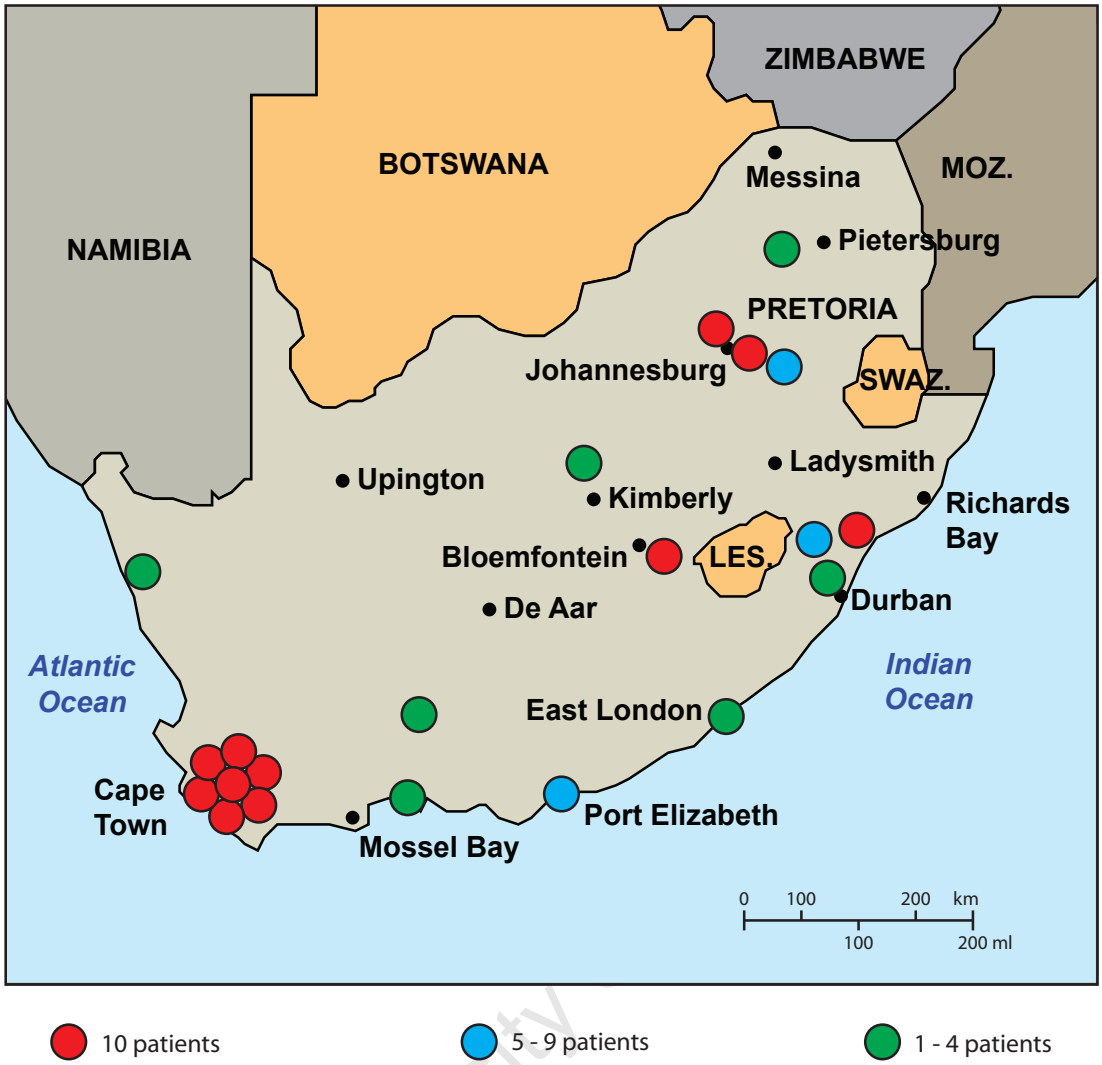

Figure 16: A schematic representation of the distribution of patients with Addison's disease in South Africa

Abbreviations:

km: kilometres

mi: miles

MOZ: Mozambique

LES: Lesotho

SWAZ: Swaziland

3.7 Clinical characteristics of Addison's disease patients

In this section, the clinical presentation, delay in diagnosis, co-morbidity at enrolment, biochemistry at diagnosis and replacement therapy will be discussed.

3.7.1 Addison's disease: clinical presentation

The clinical symptoms of the patients at presentation were elicited by interviewing them at the time of enrolment, and as such are subject to recall bias (Table 9). The most common presenting sign was hyperpigmentation (76%), followed by symptoms related to the gastrointestinal system, and with nausea and vomiting being reported by >40% of the participants. A significant number reported having suffered from loss of consciousness at presentation (20%), had a history of collapse (7%) and suffered from shock (5%). The latter symptom is the most suggestive of an Addisonian crisis. Backache occurred in 20%, while dizziness (11%) and salt craving occurred in 15%. The latter symptoms are suggestive of mineralocorticoid deficiency, but dizziness may also be a feature of GC deficiency. Non-specific symptoms such as malaise and lassitude were noted in 8% of the cohort and 3% of the cohort were noted to have hypoglycaemia. At least 7 (5%) had presented in an acute Addisonian crisis, which was suggested by a history of prior collapse, shock or hypoglycaemia. Some patients had experienced fainting spells and it was difficult to ascertain whether these episodes represented an acute Addisonian crisis. Thus, the true number of patients who presented with an Addisonian crisis may be higher than 5%.

Table 9: Addison's disease: clinical presentation

Presenting symptom	*N*	Proportion of the cohort (%)
Self reported increase in skin pigmentation	112	76
Nausea	76	51
Vomiting	63	43
Weight loss	37	25
Abdominal pain	31	21
Backache	29	20
Loss of consciousness	29	20
Diarrhoea	22	15
Salt craving	21	15
Dizziness	16	11
Malaise/lassitude	12	8
History of collapse	10	7
Shock	7	5
Hypoglycaemia	4	3
Anorexia	4	3

N: number

The proportion of 148 Addison's disease patients who manifested with any of these symptoms at presentation

A comparison of the clinical presentation with three other studies is shown in Table 10. Hyperpigmention was the most common presenting symptom in three studies, including the retrospective analysis of acute hypoadrenalism in South Africa.[8] Gastrointestinal disturbances appeared to predominate in the presentation of Addison's disease in all the studies. Weight loss only occurred in 25% of patients in this study, in contrast to the other studies, where it was far more prevalent. In this study and that of Soule, loss of consciousness and confusion were recorded as presenting symptoms, which suggest that patients with Addison's disease in

South Africa may present in a more advanced state of ill-health, compared to their first-world counterparts.[8]

Table 10: Comparison of presenting symptoms in four separate studies

South African Addison study (proportion)	Soule et al[8] (proportion)	Nerup et al[11] (proportion)	Bleicken B[12] (proportion)
South African study'	South African study	Danish study	German study
N = 148	*N* = 50	*N* = 108	*N* = 216
Hyperpigmentation (76%)	Hyperpigmentation (86%)	Weakness, fatigue, anorexia, weight loss (100%)	Fatigue (84%)
Nausea (51%)	Weight loss (67%)	Hyperpigmentation (92%)	Weight loss (66%)
Vomiting (43%)	Abdominal pain (20%)	Hypotension (88%)	Hypotension (55%)
Weight loss (28%)	Diarrhoea (16%)	Gastrointestinal disturbances (56%)	Nausea (49%)
Loss of consciousness (20%)	Confusion (14%)	Salt craving (19%)	Vomiting (44%)
Backache (20%)		Postural symptoms (12%)	Dry skin (34%) hyperpigmentation (41%)

N: number

3.7.1.1 Delay in diagnosis

Overall, the median and IQR duration of symptoms, prior to diagnosis was 5 (316) months, with a range as long as 240 months. Although a recent cross-sectional study from Germany also found a significant delay in making the diagnosis, as 20% of the subjects had had symptoms for longer than five years prior to being diagnosed,[12] only 1% of South African Addison's study patients had symptoms longer than five years prior to the diagnosis. Nevertheless, a number of case

reports attest to the prolonged interval between onset of symptoms and the diagnosis of Addison's disease.[13]

3.7.1.2 Co-morbidity reported at enrolment

As seen from Table 11, hypertension, hypercholesterolaemia and T2DM were the most prevalent CV conditions. A prior history of pulmonary tuberculosis was the most common respiratory condition. There were numerous infrequently encountered co-morbid conditions (≤1%), such as steatosis of uncertain aetiology, prosthetic cardiac valve replacement, pulmonary emboli, chronic obstructive airways disease, bronchopulmonary dysplasia, asthma, emphysema, gastro-oesophageal reflux disease, peptic ulcer disease, hepatitis, gastric atrophy, chronic renal failure, epilepsy, Bell's palsy, Duchenne's muscular dystrophy, depression, anxiety, premature menopause, prostate carcinoma, hysterectomy, polycystic ovarian syndrome, gout, rheumatoid arthritis, breast carcinoma, psoriasis, foetal alcohol syndrome, metabolic syndrome, erythrocytosis, melanomas, thyroid carcinoma and rheumatoid arthritis. The associated autoimmune conditions will be described in Chapter 4.

Table 11: Co-morbidity reported at enrolment

System	Medical condition Σ	*n/N* (%)
Cardiovascular system	Hypertension	22/148 (15)
	Type 2 diabetes mellitus Ω	9/148 (6)
	Hypercholesterolaemia	7/148 (5)
	Ischaemic heart disease	4/148 (3)
	Cerebrovascular disease	3/148 (2)
Respiratory system	Tuberculosis	11/148 (7)
Rheumatological	Osteoporosis	7/148 (5)
	Antiphospholipid syndrome	4/148 (3)
	Osteoarthritis	3/148 (2)
Other	Type 1 diabetes mellitus	11/148 (7)

n: Number of patients found to have a co-morbid illness

N: Total number of Addison's subjects

Σ: Excludes medical conditions occurring in less than 1% of the patients, for example, steatosis, prosthetic cardiac valve replacement, pulmonary emboli and chronic obstructive airways disease

Ω: Considered a cardiovascular risk factor

3.7.1.3 Biochemistry at diagnosis

As this was a retrospective study, in which the diagnosis of Addison's disease was made in several cases, 20 or more years previously, the records of the admissions were frequently unavailable. There was adequate biochemistry for the diagnosis of Addison's disease in 72% of the cases. The biochemistry was considered to be adequate by the presence of at least one of suggestive electrolytes (hyponatraemia and hyperkalaemia), low early morning cortisol and simultaneously elevated ACTH levels or an inadequate cortisol response to an ACTH stimulation test. The high proportion of patients (28%) with inadequate biochemical verification is likely to be due to the study design (Table 12). Although some patients had multiple biochemical data available, diagnostic

ACTH stimulation tests were available in 38 (26%) of the patients, early morning cortisol and simultaneously elevated ACTH levels in a further 52 (36%) patients and suggestive biochemistry in 15 patients (10%). The median basal morning plasma cortisol was 58 nmol/L. The median stimulated cortisol was 82 nmol/L, range 5.9 to 476.0 nmol/L, in all patients with complete data. Levels of ACTH >10 pmol/L were considered appropriately elevated for this range of cortisol and corroborated the presence of primary hypoadrenalism. Of the 50 patients who had a TSH measured at initial diagnosis, 13 (26%) also had primary hypothyroidism, however this may represent an overestimate as cortisol deficiency per se could induce a TSH rise, TSH ranged from (6.76-100 mIU/L). A further 2 (4%) patients had primary hyperthyroidism, (TSH < 0.02) mIU/L, by biochemical criteria.

Table 12: Biochemistry at initial diagnosis of Addison's disease

	Reference Range NHLS	*n/N* (%)	Missing data *n/N* (%)	Median	Inter-quartile range	Range
Basal cortisol (nmol/L)**	171.0 - 536.0	89/148 (59)	59/148 (40)	58.0	23.8-114	0.0–398
Stimulated cortisol (nmol/L)**	> 550	38 /148 (26)	110 /148 (74)	82.0	49.8-20.8	0–476
Plasma ACTH (pmol/L)**	1.0 - 10.1	50/148 (34)	98/148 (66)	376	178-973	12.2–1878
Serum Na (mmol/L)**	135.0 - 147.0	52/148 (35)	96/148 (65)	129.0	125-136	101–145
Frequency of hyponatraemia		35/52 (62)				
Serum K (mmol/L)**	3.5 - 5.3	49/148 (33)	99/148 (67)	5.2	4.5-5.8	3.8-8.4
Frequency of hyperkalaemia		17/49 (35)				
Renin (mU/L)**	7.0 - 76.0	22/148 (15)	126/148 (85)	62.0	24-470	12–5500
Frequency of hyperreninemia		11/22 (50)				
Aldosterone (pmol/L)**	110 - 860.0	23/148 (16)	125/148 (84)	30.9	25-139	0.09–344
Frequency of hypoaldosteronaemia		18/23 (78)				
Serum TSH (mIU/L)	0.35 - 5.5	50/148 (34)	98/148 (66)	2.43	1.07-7.83	0.01–100
Frequency of hypothyroidism		13/50 (26)				
Serum free T4 (pmol/L)	11.5 - 22.7	37/148 (25)	111/148 (75)	14.2	10.4-17.0	0.1-26.8

n: Number of patients identified with available biochemical parameter

N: Total number of Addison's patients

Plasma ACTH: plasma adrenocorticotrophic hormone

Serum Na: Serum sodium

Serum K: Serum potassium

Serum TSH: Serum thyroid stimulating hormone

Serum free T4: Serum free thyroxine

**Laboratory investigations are not mutually exclusive

NHLS: National Health Laboratory Services

Reference ranges as offered by the National Health Laboratory Services of 2011

Reference ranges likely differed as different assays were used since diagnosis.

The 105 patients with adequate or suggestive biochemistry were compared to the 43 patients with insufficient biochemical verification data (Table 13). There was no difference between these two groups in terms of age of enrolment, age of initial diagnosis and proportion that were female. The ethnic distribution and the proportion with foreign ancestry of at least a first or second degree relative were also no different. The only difference in the presenting symptoms between the two groups was that a greater proportion in the group with adequate or suggestive biochemistry presented with vomiting. There was an almost identical delay in diagnosis and there were no differences in either the prevalence of co-morbidity or replacement doses of hydrocortisone. Overall, there were substantial clinical similarities between the subjects with adequate or suggestive biochemistry and those with insufficient biochemical verification.

Table 13: Clinical characteristics of Addison's patients with adequate or suggestive biochemistry compared to those with insufficient biochemical verification

Clinical characteristics	Adequate or suggestive biochemistry	Insufficient biochemical verification	*p* - value
Number	105	43	
Age of enrolment years (IQR)	51 (35-62)	41 (21.5-54.5)	0.12
Age at initial diagnosis years (IQR)	34 (20-46.8)	30 (16.3-42.5)	0.26
Gender Female *N* (%)	 67 (64)	 24 (58)	0.46
Ethnicity *N* (%) White ancestry Mixed ancestry Asian Black	 72 (69) 23 (22) 4 (4.5) 6 (5.5)	 25 (56) 11 (26) 1 (2) 6 (14)	0.28
Foreign ancestry *N* (%)[1]	36 (34)	15 (35)	0.498
Presenting symptoms *N* (%) Pigmentation Nausea Vomiting Weight loss Abdominal pain Backache Loss of consciousness Diarrhoea Salt craving Dizziness	 77 (73) 57 (54) 51 (49) 25 (24) 22 (21) 23 (22) 23 (22) 18 (17) 16 (15) 10 (10)	 31 (72) 19 (44) 12 (28) 12 (28) 9 (21) 6 (14) 7 (16) 3 (7) 6 (14) 6 (14)	 0.72 0.36 **0.02*** 0.60 1.0 0.27 0.60 0.18 0.84 0.43
Delay in diagnosis months (IQR)	6 (3-18)	5 (2-13)	0.28
Co-morbidity at enrolment *N* (%) Hypercholesterolaemia Type 2 diabetes mellitus Hypertension Ischaemic heart disease Cerebro vascular disease Tuberculosis	 5 (5) 7 (7) 18 (17) 3 (3) 1 (0.7) 7 (7)	 2 (5) 2 (5) 4 (9) 1 (2) 2 (2) 4 (9)	 0.70 0.93 0.80 0.71 0.42 0.83
Replacement therapy Total daily hydrocortisone dose (IQR) mg	20 (20-30)	25 (20-30)	0.91

Median: age of enrolment, age of initial diagnosis, delay in diagnosis, total daily hydrocortisone dose

N: number

IQR: Inter-quartile range

1: First or second degree foreign relative from United States of America or Europe

p <0.05 considered significant

**p* <0.05

3.7.1.4 Replacement therapy

Most patients (81%) received a combination of fludrocortisone and hydrocortisone. Prednisone in combination with fludrocortisone was the next most common combination of steroid replacement administered (Table 14). The median total daily hydrocortisone dose was 20 mg and the total median dose of hydrocortisone corrected for body surface area (hydrocortisone/m^2) was 12.4 mg. In this cohort, 33% were receiving three daily doses, 52% were receiving two daily doses and 15% were receiving a single daily dose of GC replacement therapy. The proportions of patients using once-daily hydrocortisone, cortisone acetate, prednisone and dexamethasone were 12%, 66%, 40% and 100% respectively. In addition, 38% of patients reported having had at least one lifetime Addisonian crisis and 58% of patients did not wear any form of medical alert identification. The only patient who used dexamethasone as replacement therapy, was receiving a significantly greater GC exposure than any of the other patients.

Table 14: Glucocorticoid replacement therapy in South African Addison's disease patients

Preparation	*n/N* (%)	Median daily dose (IQR) (mg)	Equivalent hydrocortisone dose/kg (IQR) (mg)	Equivalent hydrocortisone dose/m² (IQR) [14] (mg)
Hydrocortisone	112 /126 (89)	20.0 (20-30.0)	0.33 (0.25-0.44)	12.4 (10.3-16.9)
Cortisone acetate	3/126 (2.0)	25.0 (25.0-32.5)	0.53 (0.50-0.55)	19.7 (18.3-21.0)
Prednisone	10 /126 (8)	8.75 (5.0-11.9)	0.42 (0.3-0.68)	11.4 (16.6-26.3)
Dexamethasone	1/126 (0.8)	5	1.31	62

n: Total number of patients identified using a specific form of glucocorticoid replacement therapy

N: Total number of Addison's patients enrolled in this analysis

IQR: Interquartile range

Hydrocortisone dose/m^2: Total daily hydrocortisone dose, corrected for body surface area

Hydrocortisone dose/kg: Total daily hydrocortisone dose corrected for body weight

Equivalent doses derived from Meikle AW and Tyler FH. Potency and duration of action of glucocorticoids. Am J of Med 1977;63(2):200-207[14]

Missing data in 22/148 (15%), patients untraceable

a: in a single patient

3.8 Discussion

There are numerous cohort studies of Addison's disease in the literature, but this is the only one conducted in sub-Saharan Africa and it can be considered to represent a large cohort as 148 patients were enrolled.[15 17] Although an extensive effort was made to identify every person with Addison's disease in South Africa, it is possible that some patients may not have been captured. Collecting the current cohort relied on referrals from primary, secondary, tertiary and quaternary centres in the country, where no databases for this disease are kept, except within the domains of the medical insurance companies. In most cohort studies, the participants were exclusively drawn from academic centres, with the exception of two reports. Willis et al[16] published data in which letters were sent to general

practitioners in the United Kingdom requesting information on patients with Addison's disease. More recently, a German survey analysed data from patients attending either an academic endocrinology department or a private endocrine practice.[12] The methods used for collecting the South African Addison's disease cohort needed to consider the major private and public health-care delivery systems. Although the majority of cases were identified from academic centres, the cohort would have been reduced by 48% if patients were not enrolled from lower levels of care and private practitioners. Nevertheless, it is quite possible that some patients were not captured, as only two-thirds of the country's medical practitioners were reached by the researcher.

Although the researcher attempted to obtain a complete biochemistry data set for each of the patients, this was not possible because in many instances patients had been diagnosed with Addison's disease up to 20 years prior to being enrolled in the study and early clinical and biochemical records of their initial admissions were not available. Biochemical data compatible with the diagnosis of primary hypoadrenalism were available for at least 72% of the patients enrolled. Nevertheless, there is no reason to doubt the diagnosis of primary hypoadrenalism, since the diagnosis was made by experienced specialist physicians (internists), paediatricians and endocrinologists and the researcher verified that the clinical picture at presentation was compatible with this diagnosis. Moreover, the clinical characteristics were similar in the group of patients with adequate biochemistry and the group with inadequate biochemical verification data (apart from a greater prevalence of vomiting in the former group). Although it could be expected that patients with insufficient biochemical data may have had Addison's disease for longer than the other group due to data being lost or discarded, this was not confirmed in this study.

Based on data from Løvås et al and Ten et al,[18 19] the prevalence of Addison's disease is estimated to vary from 39 to 117 per million, but it has been recorded as high as

144 per million.[20] Even using conservative estimates, this is considerably higher than the 3 per million found in the current study. A number of factors suggest that this cohort may not have included all of the cases of Addison's disease. Given the demographic profile of the South African population, the majority of participants would be expected to be black African, with similar numbers of white and mixed ancestry patients, and a small number of Asian patients. However, in the cohort there were small numbers of black Africans and Asians, while whites comprised the majority. The predominantly European ancestry (white) patients seen in this study may reflect that the evolution of Addison's disease is dependent on certain HLA frequencies, genetic variations or environmental factors, creating favourable conditions that promote a greater prevalence among Europeans. This may be supported by a large study from North America and the UK, which examined 2 624 vitiligo probands and found that 83% were white.[21] On the other hand, this may merely reflect the demographic profiles of these countries, since the majority of these respective populations are not black African.

Another possible explanation is that patients with Addison's disease may die with the disease unrecognised. Addison's disease is regarded as one of the mimickers in medicine. Although hyperpigmentation is a significant clinical feature in whites, it may be much more subtle in darkly pigmented races, resulting in the diagnosis being overlooked.[8 22 23] The disease may also be unrecognised due to the poor health-care system, which is challenged by the HIV/AIDS epidemic and insufficient numbers of skilled health-care workers, with some facilities having only 7% of the required number of doctors.[24] Enhancing awareness of Addison's disease may be achieved by providing annotations on chemical pathology reports and alerting clinicians to the possibility that primary hypoadrenalism may coexist with compatible biochemistry. This should be tempered by the fact that the recent audit of the Asia-Pacific region and Africa indicated that the quality of interpretive commenting was diverse, incorrect in some cases and potentially misleading.[25] There is, in addition, potential to raise awareness of Addison's disease among

medical students. This can be accomplished through so-called scripts, which are temporary mental representations, consisting of existing knowledge and new information. There are three critical elements, namely: enabling conditions (features associated with the acquisition of an illness, for example, fatigue or hereditary factors), fault (malfunctions in illness) and the consequence of faults (signs and symptoms).[26]

A significant migration of South African doctors has occurred to countries with perceived better standards of living. Analogous to the explanation for the low incidence of Addison's disease in rural settings, is the lower incidence of T1DM in rural Africa,[27] where the ratio of medical practitioners to population is considerably less than in the cities. Nevertheless, some autoimmune conditions may demonstrate a predilection for rural versus urban areas; T1DM has shown a tendency to develop in rural areas in Finland, in contrast to the African scenario.[28] The distribution of SLE has also been found to vary, even within an urban district, suggesting that geographical factors may play a role in modifying an autoimmune process. Taken together, the few black patients enrolled in the study may reflect the sub-optimal health delivery in rural areas and the particular challenge in making this diagnosis in pigmented races.

In some respects, this cohort is congruent with the findings of previous studies, for example, multiple authors have confirmed the preponderance of women in their cohorts[15 16] and the age of diagnosis is similar to many studies of adult Addison's disease.[17 29 31] As can be anticipated, the age of onset in the paediatric Addison's disease population differs substantially, as the median age of onset was 7.7 years in the study by Simm et al.[32]

Multiple authors have attested to the fact that hydrocortisone is the preferred form of replacement therapy.[19 33-35] In Italy, until 1999, cortisone acetate was the only drug available for Addison's disease.[36] Various authors have suggested that

prednisone and dexamethasone may have advantages over hydrocortisone, due to their low cortisol peaks; sustained cortisol levels potentially limiting symptoms of fatigue and the possibility of once daily administration. On the other hand, the extended half life conferred by prednisone and cortisone acetate may give rise to high non-physiological night-time cortisol levels and for this reason, hydrocortisone remains a frequently prescribed form of therapy.[33] Hydrocortisone replacement is preferred among children, administered in doses of 10-20 mg/m² in three divided doses, as it has less impact on longitudinal growth than the longer acting GC therapy.[19]

It is concerning that a significant number (58%) of patients did not wear any form of medical identification in this study, which is considerably higher than the 40% of type 1 diabetic children, aged between 6 years and 17 years of age, who did not wear medical alert identification that indicated they were diabetic and required insulin.[37] The barriers to wearing forms of medical identification were not examined, although anecdotally it is not uncommon for patients to break the chains or discs and lose their bracelets. It seems advisable to encourage the use of medical identification alerts at each clinic visit.

The patients in this study appeared to have significant co-morbidity, but it remains unclear whether this is greater than that experienced by the general population in South Africa. On the other hand, recent data from the United Kingdom indicate that aside from expected coexistent autoimmune disease, 65% of patients with Addison's disease had an elevated BMI of >25 kg/m², 65% had TC of >5 mmol/L, 17.9% had established spinal osteoporosis and 53.5% had spinal osteopaenia, corroborating the fact that Addison's patients are burdened by co-morbidity.[38]

Mortality has not been assessed in patients with Addison's disease in South Africa, but in Sweden, this has been examined by reviewing death registers. CVD causes of mortality, especially ischaemic heart disease, were twice as common

as the background population. Malignancy with no specific predilection for a specific sub-type and infectious diseases were also considerably greater than the background population. In the same study, diabetes, irrespective of the type, conferred an almost quadruple mortality in patients compared to the background population.[39]

This current study of South African Addison's disease patients has several weaknesses. As it includes a cross-sectional analysis of the clinical characteristics of Addison's disease, the diagnosis in many instances was made several years previously. This, together with the lack of uniform protocols for evaluating Addison's disease and incomplete clinical notes in a diversely functional health-care system, probably contributed to the incomplete biochemical data available. It has been noted that the prevalence of Addison's disease in South Africa is considerably lower than in Western countries. Poor access to health-care in rural regions could have accounted for this low prevalence.

3.9 Conclusions

This large cohort from sub-Saharan Africa contained small proportions of black and Asian participants, which do not reflect the overall demographics of the country. The overall prevalence of Addison's disease is considerably lower than Western countries and its diagnosis is considerably more common in urban regions that are close to quaternary teaching hospitals, compared to rural areas due to possible under-diagnosis. Raising awareness of this highly treatable condition is an important and potentially life-saving measure. In the next chapter, the aetiopathogenesis and genetic associations of Addison's disease in South Africa will be discussed.

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Chapter 4
Aetiopathogenesis and genetics of Addison's disease in South Africa

4.1 Introduction

Prior to the first half of the 20th century in developed countries, tuberculous adrenalitis was an important cause of primary hypoadrenalism. However, even today in developing countries, tuberculosis still accounts for a significant proportion of these cases.[1-3] Currently when autoimmune aetiology is assessed by adrenal autoantibodies, it accounts for the vast majority of Addison's disease in Western countries,[4-12] and tuberculous adrenalitis has become a less prominent cause.[13] South Africa is at the epicentre of HIV and tuberculosis epidemics,[14] and it is uncertain whether the background prevalences of these communicable diseases influence the underlying aetiology of Addison's disease.

A single study of acute Addison's disease from South Africa suggested an underlying autoimmune aetiology of only 12%, with the majority of cases being idiopathic (42%) or tuberculosis related (34%). However, adrenal autoantibodies were not measured.1 T1DM has been extensively studied and serves as a prototype for the study of other autoimmune conditions. Islet-cell autoantibodies (ICA) titres decline following diagnosis and only 5 10% of T1DM patients remain ICA positive ten years after diagnosis. Thus, it is likely that a similar pattern may be seen in patients that were diagnosed with Addison's disease many years previously, with a significant number of patients being adrenal autoantibody negative.[15] People of African origin may also have a different autoantibody profile when compared to European patients, as only 4 in 10 African-Americans are ICA positive, with new onset T1DM.[15-17]

The aim of this study was to examine the aetiology of Addison's disease in a

large South African cohort, with the hypothesis that autoimmunity would be more common than previously described in the country. In addition, since HLA DQ alleles have previously been reported to associate with adrenal autoimmunity, especially in APS2 with Addison's disease and T1DM,[18 19] the cohort was examined to determine whether there was a predisposition to autoimmunity, based on HLA DQB1 genotypes.[20]

4.2 Patients and methods

The methods employed in this cross-sectional study design are outlined below.

4.2.1 Patients

The patients enrolled in the Addison's disease cohort (described in Chapter 3) were eligible for inclusion in this study.

At enrolment, all patients had blood drawn for ACA, 21-hydroxylase autoantibodies, islet cell autoantibodies (ICA), thyroid microsomal antibodies, thyroglobulin autoantibodies (anti-Tg), tissue transglutaminase autoantibodies (anti-TTG), StCAb (ovarian, testicular and placental antibodies) and parietal cell antibodies. All male patients had VLCFAs evaluated for confirmation or exclusion of ALD. In addition, HLA DQB1 genotypes were determined for each patient and healthy control subject that attended the blood donor clinic. Patients and control subjects were matched for gender and ethnicity.

4.2.2 Measurement of serum autoantibodies

Autoantibodies expected to occur in association with autoimmune Addison's disease were assayed as discussed below.

4.2.2.1 Adrenocortical autoantibodies (ACA)

TThese were determined using indirect immunofluorescence on cryostatic

sections of human adrenal glands.[21 22] Within two hours of surgical removal, the substrate of human adrenal gland was snap frozen in isopentane, which had been cooled in a dry ice acetone bath. This was performed by the Department of Pathology, University of Florida, Gainesville, USA.

4.2.2.2 21-hydroxylase autoantibodies

21-hydroxylase autoantibodies were measured with a commercial immunoprecipitation assay based on ^{125}I-labeled recombinant human 21-OH (RSR Ltd., Cardiff, UK). 21-hydroxylase autoantibody levels above 1 U/ml were considered positive.[23] The assay has high specificity for 21-hydroxylase antibodies and is more sensitive than immunofluorescence assay for detecting adrenal antibodies in cases with overt adrenal failure.[24]

4.2.2.3 Islet-cell autoantibodies (ICA)

Islet-cell autoantibodies (ICA) were determined by the Department of Pathology in the University of Florida, Gainesville, USA in an indirect immunofluorescence assay, using cryostatic sections of human pancreas from patients with blood group O. Values of ≥10 Juvenile Diabetes Foundation (JDF) units were considered positive. In past Immunology of Diabetes Society (IDS) workshops, this ICA assay had a specificity of 100% and a sensitivity of 74.4% in patients with new-onset T1DM, who were less than 30 years of age.[25 26]

4.2.2.4 Thyroid microsomal autoantibodies

Thyroid microsomal antibodies were measured using the kit, employing haemagglutination methodology, Thymune-M (Remel Europe Ltd, Dartford, Kent, UK). Results were considered positive if the titre exceeded 1:400.

4.2.2.5 Thyroglobulin autoantibodies

Thyroglobulin autoantibodies (anti-Tg) were measured using the kit, employing haemagglutination methodology, Thymune-T (Remel Europe Ltd, Dartford, Kent, UK). Results were considered positive if the titre exceeded 1:100.

4.2.2.6 Tissue transglutaminase autoantibodies

Tissue transglutaminase autoantibodies (anti-TTG) IgA antibodies against tissue transglutaminase were determined with a routine in-house ELISA assay at the Department of Clinical Immunology, University Hospital, Uppsala, using commercially available guinea pig transglutaminase and rabbit anti-IgA antibodies. Microplates were coated with Sigma tissue transglutaminase or human tissue transglutaminase. After incubation plates were blocked with Tris-hydrochloride and washed. Patient sera were diluted and horseradish peroxidase-conjugated rabbit anti-human IgA antibodies were added. A substrate solution containing 1,2-phenylenediamine-dihydrochloride and H_2O_2 was added. The absorbance was measured at 492 nm. The upper normal reference range of the 95th percentile was 6 kUnits/L.

4.2.2.7 Steroid cell producing autoantibodies

Steroid cell producing autoantibodies (StCA) were measured at the Department of Pathology at the University of Florida by indirect immunofluorescence, using human placenta, human testes and pregnant rabbit ovary as substrate. StcA may react with syncytiotrophoblast in the placenta, Leydig cells in the testes, and/or the theca interna/granulosa cell layers of the Graafian follicles, and the corpora lutea of the ovaries. Some StcA-positive sera react with all tissues, whereas other sera may only react with one or two tissues.[27]

4.2.2.8 Parietal cell antibodies

Parietal cell antibodies were measured at the Department of Pathology at the University of Florida by indirect immunofluorescence, using human stomach as substrate and read as positive or negative.[28]

4.3 Very Long Chain Fatty Acids (VLCFAs)

The plasma VLCFA concentration was determined by capillary gas chromatography mass spectrometry, following procedures described previously.[28 29] Normal values were previously determined in 50 healthy South African controls that were similar in age to the study population, {C26:0 (0.5-2.1µmol/L), C24:0 (12.6-46.0µmol/L) and C22:0 (33.9-157.5µmol/L}. Normal C24/C22 and C26/C22 ratios were calculated as 0.2-0.4 and 0.01-0.02 respectively. All abnormal results obtained were confirmed by an external laboratory (Lab. Genetic Metabolic Diseases, F0-225, Academic Medical Centre, University of Amsterdam, The Netherlands).[29 30]

4.4 HLA DQB1 genotyping and aetiological classification

HLA DQB1 genotypes were determined using a previously described method.[32] Briefly, exon 2 of the DQB1 gene was amplified using polymerase chain reaction (PCR). The amplified PCR products were then separated by denaturing gradient gel electrophoresis (DGGE). DQB1 alleles were determined by comparing the migration of bands from samples to standards with known identity. Individuals with DQB1* 0302.3 or DQB1* 0602.3.4 alleles on DGGE were further typed using sequence specific primer techniques. Alleles *0302 and*0303 were distinguished using a DQB1*0302 specific amplification. The DQ6 alleles were distinguished using three sequence-specific primers.

The aetiological classification of Addison's disease is demonstrated in Table 15. Autoimmune Addison's disease was diagnosed when subjects were positive for ACA and/or 21-hydroxylase autoantibodies. Tuberculosis-related adrenal insufficiency was diagnosed if there was a prior or current history of tuberculosis.[8] Biopsy-proven or radiological evidence for metastatic disease indicated a metastatic aetiology of Addison's disease, while patients with sarcoidosis or iron overload and no other clear cause of hypoadrenalism had Addison's disease attributed to these conditions.[33] ALD was diagnosed as a cause of hypoadrenalism if the VLCFAs were increased,

irrespective of the autoantibody pattern.[34 35] X-linked adrenal hypoplasia was diagnosed in boys with primary adrenal failure and salt loss in the first few weeks of life, which is associated with hypogonadotrophic hypogonadism. The diagnosis of ACTH-resistance syndrome was made based on primary hypoadrenalism and isolated GC deficiency, but with normal mineralocorticoid function.[36 37] Patients with an AIDS-defining illness were classified as having AIDS-related Addison's disease. The diagnosis of idiopathic Addison's disease was reserved for patients in whom there was no obvious clinical cause and had no adrenal autoantibodies.6 Sarcoidosis, iron overload and HIV were excluded clinically, using the appropriate laboratory studies.

Table 15: Aetiological classification of Addison's disease

Aetiology	Diagnostic Criteria
Autoimmune Addison's disease	Presence of 21-hydroxylase autoantibodies or ACA
Adrenoleukodystrophy	Increased plasma VLCFA, irrespective of autoantibody pattern
Tuberculosis	A compatible current clinical or past history of tuberculosis of lung, bone, pelvic-peritoneal or genitourinary; radiology compatible with tuberculosis
AIDS (related)	Presence of an AIDS defining illness for example cytomegalovirus, *Mycobacterium avium- intracellulare, Cryptococcus neoformans, Toxoplasmosis gondii*, Kaposi's sarcoma
Other causes	Malignancy (biopsy proven), sarcoidosis, iron overload
X-linked Adrenal hypoplasia	Male patients with primary hypoadrenalism, salt loss in the first few weeks of life, and later frequently associated with hypogonadotrophic hypogonadism
ACTH resistance syndrome	Isolated glucocorticoid insufficiency, but normal mineralocorticoid function, may be associated with alacrimia, achalasia
Idiopathic	Adrenal autoantibody negative, normal VLCFAs, no history of tuberculosis or genetic form

VLCFA: Very long chain fatty acids
AIDS: Acquired immune deficiency syndrome
ACTH: Adrenocorticotrophic hormone
ACA: Adrenocortical autoantibodies

4.5 Statistical methods

Patient characteristics with non-normal distribution were described using the median and IQR for continuous variables and numbers of patients, and percentages for binary and categorical variables. Data were compared between autoantibody groups using the univariate linear regression Wald test for continuous variables, and Chi-squared tests for binary and categorical variables.

The relative risk for autoimmune Addison's disease, compared to controls in HLA allele frequency, were described for European ancestry (white) patients without T1DM, in order to avoid any confounding that may arise due to ethnicity or known associations with T1DM. Factors associated with an autoimmune aetiology in the study cohort were explored in univariate and multivariate logistic regression models.

The 21-hydroxylase autoantibody titres were transformed to a logarithmic scale, plotted and regressed against the duration of Addison's disease at enrolment in a two-way scatter-plot. All analyses were conducted using Stata(TM) version 10.0 (Stata Corp., College Station, Texas).

4.6 Results

As seen in Figure 17, 144 of the 161 patients referred for enrolment in the South African Addison's study were included in the study of aetiopathogenesis and genetics. The clinical characteristics of the patients enrolled in the study and the seven who were excluded because they were too late for enrolment, are shown in Table 16. The median and IQR age of enrolment was 46.5 (32.8-61.0) years and the range was 2.7-88.0 years. The age at initial diagnosis was 34.0 (20.0-45.0)

years, indicating that most patients had had the disease for a long period of time (12.5 years; range 0-50 years). There was a greater preponderance of women, who constituted 61% of the cohort. Most patients (n = 109, 75%) were over the age of 20 years at diagnosis and 49% had been diagnosed more than ten years earlier. 88% of patients were recruited from the major cities, often in close association with a major teaching hospital. The ethnicity of the cohort was heterogeneous: 65% European ancestry (white), 24% mixed ancestry, 3% Asian and 8% were black South Africans.

The seven patients who were not included in this study differed from the 144 who were enrolled. They were younger at initial diagnosis (17.0 years versus 34.0 years; p = 0.048), had been suffering from Addison's disease for a shorter period of time (they had predominantly either new-onset Addison's disease or had been diagnosed between 1.0 and 4.9 years ago), were lighter (53.1 kg versus 70.0 kg; p = 0.04) and had a lower BMI (21.2 kg/m^2 versus 25.2 kg/m^2; p = 0.01). This information is summarised in Table 16.

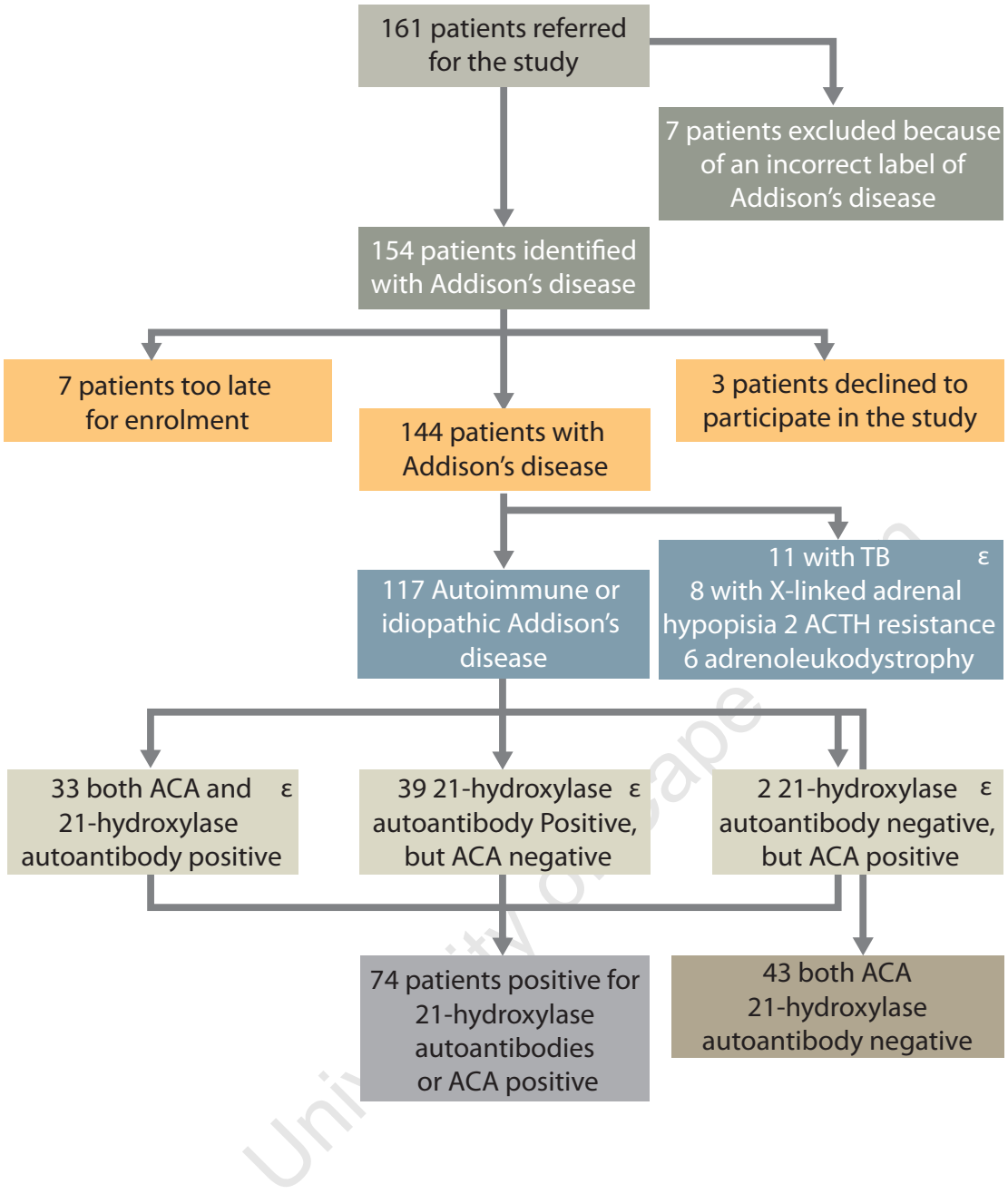

Figure 17: Diagnostic categories of patients enrolled in the South African Addison's disease study for evaluating underlying aetiopathogenesis and genetics

TB: Tuberculosis

X-linked: X-linked congenital adrenal hypoplasia

ACTH resistance: Adrenocorticotrophic hormone resistance syndrome

ACA: Adrenocortical autoantibodies

ε: Identifiable causes of Addison's disease in 101 patients

Table 16: Clinical characteristics of Addison's disease subjects enrolled and seven subjects too late for inclusion in the study

Patients		Patients enrolled *N* = 144	Patients too late for enrolment *N* = 7	*p* - value Comparison between subjects enrolled and subjects enrolled after recruitment ended
Age at enrolment years	median (IQR)	46.5 (32.8 -61.0)	38.5 (19.0-59.0)	0.30
Age at initial diagnosis years	median (IQR)	34 0 (20.0-45.0)	17.0 (4.0-34.0)	**0.048***
Duration of Addison's disease at enrolment	New onset 1-4.9 years 5.0-9.9 years ≥ 10 years	*N* (%) 15 (10) 30 (21) 28 (19) 71 (49)	*N* (%) 3 (43) 2 (29) 1 (14) 1 (14)	**0.03***
Gender	Female	*N* (%) 88 (61)	*N* (%) 4 (57)	1.0
Height, mass and BMI at enrolment*	median (IQR) Mass (kg) Height (cm) BMI (kg/m²)	 70.0 (60.0-83.5) 165.5 (160.0-170.0) 25.2 (22.4-30.4)	 53.1 (40.6-65.0) 166 (147.3-172.0) 21.2 (20.0-23.0)	 **0.04*** 0.1 **0.01***
Ethnicity	White ancestry (European) Mixed ancestry Asian Black	*N* (%) 94 (65) 34 (24) 5 (03) 11 (08)	*N* (%) 5 (71) 1 (14) 0 (0) 1 (14)	0.74
Pigmentation at presentation	Self-reported increase	*N* (%) 108 (75)	7 (100)	0.20
Most frequent reported family history**	Addison's disease Type 1 diabetes mellitus Hypothyroidism Graves' disease	*N* (%) 11 (8) 11 (8) 10 (7) 8 (6)	*N* (%) 2 (29) 2 (29)	0.41

Patients		Patients enrolled *N* = 144	Patients too late for enrolment *N* = 7	*p* - value Comparison between subjects enrolled and subjects enrolled after recruitment ended
Aetiology	 ACTH resistance ALD Autoimmunity Idiopathic Tuberculosis X-linked adrenal hypoplasia	*N* (%) 2 (1) 6 (4) 74 (51) 43 (30) 11 (8) 8 (6)	*N* (%) 4 (57) 3 (43)	0.9

N: Number

Mass available for 107 patients, height and BMI for 94

**: Categories are not mutually exclusive

CI: Confidence interval

BMI: Body mass index

ACTH: Adrenocorticotrophic hormone

ALD: Adrenoleukodystrophy

p <0.05 considered significant

**p* <0.05

As seen in Figure 17, 70% of the cohort (n = 101) had identifiable causes of Addison's disease. Of these, 51% had autoimmunity based on the presence of 21-hydroxylase and/or ACA, 8% had a history of tuberculosis, 4% had ALD, 1% had ACTH-resistance syndrome and 6% had X-linked adrenal hypoplasia. A presumptive diagnosis of tuberculous adrenalitis was made in 8% of the patients. Only two of the patients had CT scans and the results from both were suggestive of tuberculous adrenalitis. In 30%, no specific aetiology was uncovered. There were no patients with sarcoidosis, iron overload, metastatic disease or AIDS-related Addison's disease. As ALD and X-linked AHC occur almost exclusively in males the true frequency among males is 11% and 14% respectively.

There were 111 patients (74%) with at least one associated autoimmune condition. Of these, 44 (29%) had two, 16 (11%) had three and 6 (4%) had four associated autoimmune conditions. Of the 74 patients with at least one adrenal autoantibody, 72 (96%) had autoantibodies to 21-hydroxylase, 35 (48%) had ACA and 33 (45%) were positive for both antibodies (Table 17). Of the 74 patients with adrenal autoimmunity, clinical primary hypothyroidism was the most commonly associated autoimmune condition (47%), while POF (8%) and T1DM (7%) were the next most prevalent disorders. Hypoparathyroidism, pernicious anaemia, coeliac and Graves' disease, immune thrombocytopenic purpura, ulcerative colitis, and vitiligo coexisted in 5% or fewer of the patients who were positive for 21-hydroxylase and/or ACA. As expected, the presence of associated autoantibodies did not always correlate with a concurrent clinical autoimmune condition in the study. For example, in the patients with adrenal autoantibodies, primary hypothyroidism was present in only 20 of the 32 patients with positive thyroid microsomal, coeliac disease was present in 2 of the 6 patients with positive anti-TTG, pernicious anaemia was diagnosed in 2 of the 11 patients with positive anti-parietal cell antibodies and although 6 patients had T1DM, only 4 of these patients had positive ICA. This suggests that this sub-group is at risk of future coexistent autoimmune conditions.

The proportions of the total 144 patient cohort with either APS1 or APS2 were 5 (3%) and 66 (46%) respectively. A positive family history for autoimmune disease was noted in 26% of this cohort. A positive family history for Addison's disease and T1DM occurred most frequently in 8% of patients, 10 (7%) of the patients had a first-degree relative with primary hypothyroidism disease, and Graves' disease occurred in 5% of patients' relatives, while pernicious anaemia (1%), vitiligo (0.7%), SLE (1%) and rheumatoid arthritis (1%) were less commonly encountered in the patients' relatives.

When comparing Addison's disease patients with autoimmunity to those defined as idiopathic (Table 17), the former were younger at diagnosis and were more

likely to have a positive family history of autoimmune disease. However, among the idiopathic Addison's disease group, 20 patients (47%) had at least one other organ-specific autoantibody. It is possible that some of these subjects had lost serologic evidence of adrenal autoimmunity over time, and adding this group to the autoimmune group would increase the proportion of patients with autoimmune Addison's disease in the entire cohort to 65%. The length of time for which the patients had the disease at enrolment was not different between the idiopathic group without antibodies and the autoimmune group.

The idiopathic group was older than the other two groups comprising the presence of at least one adrenal autoantibody and the group with an identifiable cause of Addison's disease excluding autoimmune, ($p = 0.002$). Interestingly, none of the 5 Asian or 11 black patients was positive for adrenal autoantibodies. Although more patients with adrenal autoantibodies had a positive family history of autoimmune disease and had a history of foreign ancestry (a first- or second-degree relative born in Europe or USA), the latter did not reach statistical significance. The mean total daily hydrocortisone replacement dose was higher in the autoimmune group (24.8 mg) compared to the idiopathic group (21.0 mg).

Table 17: Comparisons based on adrenal autoantibody status

	ACA (+) and/or anti-21 (+) (Autoimmune)	ACA (-) and anti-21 (-) (Idiopathic)	*p* - value
Patients in group (*N*)	74	43	
Gender *N* (%) Female	54 (73)	30 (70)	0.71
Age at diagnosis Age in years (95% CI)	33.1 (29.3-36.9)	40.0 (34.6-45.5)	**0.03***
Duration of Addison's disease at enrolment *N* (%) New onset 1-4.9 years 5-9.9 years ≥ 10 years	 6 (8) 18 (24) 13 (18) 37 (50)	 7 (16) 9 (21) 6 (14) 21 (49)	0.57
Ancestry *N*(%) European Mixed Ancestry Asian Black African	 65 (88) 9 (12) 0 (0) 0 (0)	 21 (49) 15 (35) 2 (05) 5 (12)	**<0.001***
Other antibodies *n/N*(%) Any other autoantibody Transglutaminase GADA† Thyroid microsomal Anti-thyroglobulin Parietal cell Islet Cell Ovarian Testicular Placental	 46/74 (62) 4/73 (5) 15/71 (21) 24/74 (32) 8/74 (11) 11/74 (15) 4/74 (5) 5/74 (7) 1/74 (1) 1/74 (1)	 20/43 (47) 2/43 (5) 9/43 (21) 2/43 (5) 9/43 (21) 3/43 (7) 2/43 (5) 0/43 (0) 3/43 (7)	 0.1 0.85 0.18 0.25 0.4 0.73 0.64 0.44 0.11
Prevalence of other autoimmune diseases *N* (%) Any autoimmune disease	44 (59)	27 (63)	0.72
Family history and ancestry *N* (%) Family history Foreign ancestry‡	 24 (32) 36 (49)	 5 (12) 13 (30)	 **0.01*** 0.05
Therapy-mean (mg) (95% CI) Hydrocortisone dose Hydrocortisone dose/m^2 Fludrocortisone dose Fludrocortisone dose/m^2	 24.8 (22.8-26.8) 16.2 (11.2-21.2) 0.1 (0.1-0.1) 0.1 (0.0-0.1)	 21.0 (18.6-23.5) 11.4 (9.5-13.4) 0.1 (0.1-0.1) 0.1 (0.0-0.1)	 **0.03*** 0.31 0.90 0.64

ACA: Adrenocortical autoantibodies

Anti-21: 21-hydroxylase autoantibodies

GADA: Glutamic acid decarboxylase 65 antibodies

CI: Confidence interval

mg: Milligram

Univariate linear regression Wald test: used for continuous variables, Chi-squared statistic for proportion

†: Only done in 21-hydroxylase autoantibody positive patients

‡: Presence of autoimmune disease in first-degree relative

$p < 0.05$ considered significant

Hydrocortisone dose: Total daily hydrocortisone dose

Fludrocortisone dose: Total daily fludrocortisone dose

Hydrocortisone dose/m²: Total daily hydrocortisone dose in relation to total body surface area

Fludrocortisone dose/m²: Total daily fludrocortisone dose in relation to total body surface area

An inverse correlation was present between titres of 21-hydroxylase autoantibodies and duration of disease since diagnosis (Figure 18). The duration of Addison's disease does not appear to account for the absence of adrenal autoantibodies in the Asian and black sub-groups, as it was no different compared to the remaining ethnic groups ($p = 0.59$), albeit that the former sub-groups were small.

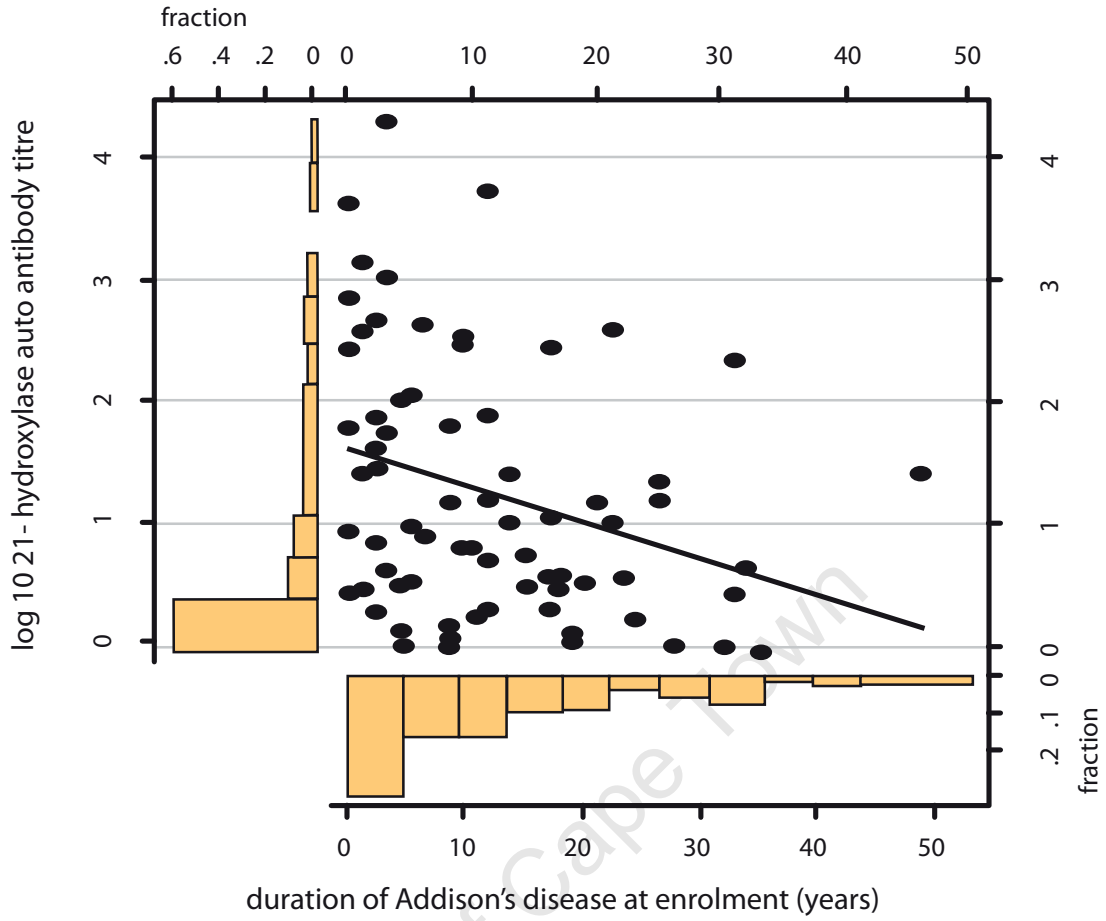

Figure 18: Lowess (locally weighted scatterplot smoothing) plot showing the relationship between duration of Addison's disease and log transformed titre of 21-hydroxylase autoantibodies. The histograms indicate a skewed distribution of antibodies, with the greatest number of patients having the lowest antibody titre (y-axis) and shortest duration of Addison's disease at enrolment.

4.6.1 HLA alleles and autoimmune Addison's disease

Compared to the controls, the HLA DQB1*0201 allele was positively associated with autoimmunity in European ancestry (white) patients without T1DM, while the *0601 allele was negatively associated (Table 18). All patients with T1DM had the alleles *0201, *0302 or *0301. Hence, these patients were excluded to avoid bias. In sensitivity analyses, these associations persisted in the entire cohort, and due to the small numbers, could not be adequately described in patients of mixed, black or Asian ancestry. When comparing all the patients with autoimmune Addison's disease to all the other patients with Addison's disease, the same crude

associations were present.

Table 18: Associations between autoimmune Addison's disease and HLA subtypes

Allele	Tested (*N*)	Autoimmune Addison's disease present *N* (%)	Controls present *N* (%)	Prevalance ratio	*p* - value[1]
0201	55	36 (65)	26 (43)	1.5	**0.02***
0302	55	20 (36)	17 (28)	1.3	0.36
0301	55	13 (24)	21 (35)	0.7	0.18
0402	55	8 (15)	8 (13)	1.1	0.85
0500	55	10 (18)	17 (28)	0.6	0.2
060x	55	15 (27)	22 (37)	0.7	0.30
0601	55	1 (2)	7 (12)	0.2	**0.03***

1 Comparison of alleles in patients with autoimmune disease and healthy control subjects
$p < 0.05$ considered significant

4.6.2 HLA DQB1 genotypes and autoimmune Addison's disease

The most common genotypes in all the patients with Addison's disease were *0201/*0302 (19%) and *0201/*060x (10%). The *0201/*0302 genotype was more common in patients with autoimmune Addison's disease (28%) compared to controls without Addison's disease (8%; $p = 0.002$). This was also the only genotype associated with autoimmune Addison's disease when compared to patients with Addison's disease of other aetiology (unadjusted odds ratio [OR] 3.2, 95% CI 1.3–8.3; $p = 0.01$). This information is summarised in Table 19.

None of the HLA alleles or heterozygous genotypes were significantly associated with autoimmune aetiology in a multivariate analysis that was adjusted for sex and ancestry. However, there was some evidence for a potential association with the

*0201/*0302 heterozygous genotype (adjusted odds ratio [AOR] 2.4; $p = 0.105$) as shown in Table 19.

Table 19: Associations with autoimmune Addison's disease

	Univariate analyses[n]			Multivariate analyses		
Characteristic	OR	95% CI	*p* - value	OR	95% CI	*p* - value
Female gender	2.9	1.4-5.7	**0.003***	3.0	1.2-7.1	**0.02***
Age of diagnosis (per 10 year increase)	1.0	0.9-1.2	0.86			
European ancestry (white)	10.2	4.4-23.7	**<0.001***	10.1	3.9-25.8	**<0.001***
Any other associated autoimmune disease	2.1	1.1-4.0	**0.03***			
Family history of autoimmune disease	4.3	1.7-10.8	**<0.001***			
Foreign ancestry	2.5	1.3-5.1	**0.008***			
Pigmentation at diagnosis	2.3	1.1-5.0	**0.03***	2.5	0.9-6.9	0.07
Severe presentation	2.6	1.3-5.2	**0.005***			
Any other autoantibodies	3.6	1.7-6.7	**<0.001***	2.3	1.0-5.4	**0.045***
Presence of HLA types 0201 0302 0301 0402 0500 060X 0601	 2.6 2.0 1.0 2.5 0.8 0.8 0.7	 1.2-4.7 0.8-3.6 0.4-1.6 0.5-5.5 0.2-1.3 0.3-1.3 1.3-8.8	 **0.01*** 0.13 0.49 0.07 0.15 0.22 **0.007***			
Presence of specific HLA combination 0201/0302	3.2	1.3-8.3	**0.01***	2.4	0.8-7.1	0.1

OR: Odds ratio

CI: Confidence interval

n = 117 and excludes patients with known aetiology of Addison's disease other than autoimmunity

HLA: Human leukocyte antigen

$p < 0.05$ considered significant

4.7 Discussion

This study is the largest cross-sectional study of Addison's disease conducted in sub-Saharan Africa in a heterogeneous population of predominantly European descent (white ancestry). The study refutes an earlier report suggesting that autoimmunity is an uncommon aetiology for Addison's disease in South Africa.[1] However, autoimmunity was previously assessed without the use of specific adrenal autoantibodies. It has been shown that ACA are not nearly as detectable as 21-hydroxylase antibodies when monitoring Addison's disease a long time after its onset. An inverse relationship exists between the length of time that a patient has had Addison's disease and 21-hydroxylase antibody titres. Curiously, none of the Asian or black subjects, although both groups did have small numbers, demonstrated adrenal autoantibodies. It is likely that Addison's disease is being under-diagnosed in South Africa, with potential grave consequences to some patients.

Over 50% of our cohort was classified as autoimmune on the basis of at least one positive adrenal autoantibody. If one considered long-standing Addison's patients who were adrenal autoantibody negative, but had other autoimmune clinical conditions, as likely to have autoimmune Addison's, then the prevalence of autoimmune Addison's rose to 65%. There was an inverse correlation between the titres of 21-hydroxylase autoantibodies and the duration of the disease since diagnosis, which confirmed that the adrenal autoimmunity wanes with increasing time after diagnosis. Thus, consistent with Western studies, this study confirms that autoimmunity is the most common cause of Addison's disease in South Africa in a cohort that was predominantly of European descent (white ancestry).[5] On the other hand, very few black patients were enrolled and none of them had autoimmunity, which may explain the similarity in prevalence to Western countries.

The cross-sectional design and the measurement of autoantibodies, in many cases long after the initial diagnosis, are the major limitations of this study. Previous

studies have demonstrated a 20% decline in the prevalence of autoantibodies in subjects followed for two or more years after the diagnosis of Addison's disease.[5 9] This study clearly demonstrates that 21-hydroxylase autoantibody titres decline with increasing duration of Addison's disease. Therefore, the possibility exists that patients who were adrenal autoantibody positive at diagnosis were antibody negative at enrolment in the study, thus falsely reducing the reported prevalence of autoimmune Addison's. A further limitation is that very few patients underwent a CT scan of the adrenal glands, an investigation of proven diagnostic utility, particularly in the setting of negative autoantibodies. The final limitation of the study relates to possible ascertainment bias. Although every effort was made to comprehensively identify patients with Addison's disease, it was impossible to determine what proportion of cases were missed due to non-response, or whether there was any selection bias in the patients included in the cohort. It is relevant that the vast majority of the cohort were of European ancestry (white ancestry), and a minority were black African and Asian. This is in contrast to the demographics of the South African population, in which the majority are black Africans (79%) with smaller proportions of European ancestry (white ancestry) (10%), mixed races (9%) and Asians (2%). It is unclear whether the preponderance of European (white ancestry) patients with Addison's disease represents a true higher prevalence of the disease in this population, or whether it is an artefact reflecting the more limited access that the black population has to health-care, with the result that there may be missed diagnoses and/or under-reporting.

The prevalence of tuberculosis and other known causes of Addison's disease (8% and 11% respectively in this cohort), are in agreement with European (white) studies.[5 11] It should be noted that the prevalence of TB in this study is 8%, which is considerably greater than the 1% background prevalence in South Africa. However, India is also a developing country like South Africa, and in a North Indian cohort with adrenal insufficiency, the prevalence of primary adrenal failure attributable to tuberculosis was considerably greater (50%) than that found in this study (8%).[2] In

the North Indian cohort of 38 Caucasians, the diagnosis of tuberculosis was made on the basis of enlarged or calcified adrenal glands seen by abdominal imaging. If the adrenal glands were enlarged, cytology and microbiological examination (inter alia Zeil Nielsen staining) were performed, as well as radiology of the chest in order to exclude pulmonary tuberculosis. Granulomatous disease, indicative of either tuberculosis (n = 18) or fungi (n = 1), was diagnosed in 19 out of the 38 patients. In the remaining 19 idiopathic patients, 4 (21%) had positive 21-hydroxylase autoantibodies, which were no different from those with granulomatous disease. Therefore, the prevalence was considerably lower than in the South African Addison's study, where 50% had positive 21-hydroxylase autoantibodies. Moreover, as abdominal imaging was not routinely performed in the South African Addison's disease study, it is possible that Addison's disease attributable to tuberculosis could have been underestimated. This difference in the prevalence of 21-hydroxylase autoantibodies, may suggest that North Indian Caucasians could have had different HLA class II antigens, compared to patients with Addison's disease from Western countries.[2] The multivariate analysis in the South African Addison's study, demonstrated that European ancestry (white) had an OR of 10.1, emphasising the likely similarity between the South African cohort and European cohort studies.

There have been a few other cohort studies from developing countries. For example, in a Tunisian paediatric population with Addison's disease, 3 out of 6 patients had Allgrove's syndrome, there was APS1 in one patient, ALD in one patient and the underlying aetiology could not be determined in the remaining patient.[38] In another cohort from India, similar to that reported by Nigam et al, [2] 47% of the patients had Addison's disease attributable to tuberculosis. 17% of these patients had positive adrenal autoantibodies, indicating the potential for overlap in these two conditions.[3] In a small study from Ethiopia, in which three Addison's patients were identified, two were considered to have autoimmune adrenal disease.[39]

It is conceivable that if the population of black patients in the South African Addison's

study were higher, then the proportion of cases due to autoimmunity may have been different. There are several reports indicating that black South Africans have a reduced, but not negligible risk of developing autoimmune disease. In the absence of antibody confirmation, African subjects are at definite risk of developing T1DM, with incidences ranging from 1.5/100 000 in Tanzania to 20/100 000 in Morocco.[40] Initial reports of rheumatoid arthritis prevalence in black Africans indicated that it was a disease that rarely occurred. However, recently, there is evidence that its prevalence is increasing, but not to the level that it occurs in Western societies.[41] The incidence of SLE among black South Africans has been reported at 12.2/100 000, which is significantly higher than among Brazilians at 8.7/100 000, but is less than 19.7/100 000 for Saudi Arabians and 60/100 000 identified in Hong Kong.[42] Based on these data, which are in contrast to the findings of the South African study in which none of the black and Asian patients were found to have autoimmune Addison's disease, it is highly likely that with increasing awareness for autoimmune Addison's disease, it will be diagnosed more frequently in these two ethnic groups.

Given the high background prevalence of tuberculosis and HIV (tuberculosis case finding was 1 000/100 000 and antenatal HIV seroprevalence was 29% in South Africa in 2006), surprisingly few patients presented with either tuberculosis or HIV-related Addison's disease in this study.[43 44] However, an earlier study from a South African tertiary care hospital, determined that primary hypoadrenalism is uncommon in a cohort of acutely ill, hospitalised patients with active pulmonary tuberculosis. This supports the contention that tuberculosis-related primary adrenal failure is relatively uncommon.[45]

The heterogeneity of the population group studied is likely to have contributed to the observed frequencies of adrenal autoimmunity. Consistent with most studies, the vast majority of these patients were of European ancestry (white ancestry), with black and Asian participants constituting a small minority. None of the black and Asian patients had adrenal autoantibodies. Similarly, black Africans with true T1DM

in sub-Saharan Africa have decreased frequencies of ICA, compared with their European ancestry counterparts. This suggests that there are genetic differences between these populations for the lack of antibody response in both T1DM and Addison's disease, or that the aetiologies of these disorders are considerably different.[16 17]

It is expected that 50% of patients with autoimmune Addison's disease will have coexistent APS. In a group of 239 Italian patients with autoimmune Addison's disease, 41% were thought to have APS2, while 13% had criteria for APS1. The proportion of APS1 was 11% in an Italian cohort of 317 individuals with Addison's disease, irrespective of the aetiology. This is significantly greater than the 3% recorded in the South African Addison's disease patients. Addison's disease is often associated with other autoimmune diseases, including T1DM and hypothyroidism, making it difficult to determine whether the observed HLA associations are due to the presence of other autoimmune diseases or to Addison's disease specifically.[46 47] This South African Addison's study excluded patients with autoimmune Addison's disease and associated autoimmune conditions to eliminate this source of bias. The sample sizes in almost all studies are small, limiting study power and increasing the chance of a type 2 error. On the other hand, the observed associations could reflect random chance. In the regression analysis, a positive association between Addison's disease and several HLA*DQB1 alleles, especially DQB1*0201 and *0302, and *0601 alleles and the *0201/*0302 genotype, was observed. The presence of T1DM in patients with APS2 is reportedly strongly associated with DQB1*0201 and DQB1*0302 alleles. However, even after excluding Addison's patients with T1DM, an association with *0201 and *0302 persisted, confirming a previous report.[48] The DQB1*0201 frequency of 40% in the healthy population is extremely high and raises concerns about the genotyping quality. Even though this study has a relatively large sample size compared to some previous studies, the study power is still limited and associations require confirmation in further larger studies.

In contrast to this study, 21-hydroxylase autoantibodies titres did not decline with increasing duration of the disease among North Indian Caucasians.[2] However, these patients were studied for a much shorter time {mean duration of Addison's disease was 12 months (range 1-72 months), compared to 13.3 years (range 0.03-50 years)} in the South African Addison's disease study. 21-hydroxylase autoantibodies were twice as detectable, compared with ACA. Moreover, the clinical associations appear to be stronger with 21-hydroxylase autoantibodies than ACA, suggesting that the former antibody may be a far more reliable marker of autoimmunity than ACA a long time after the diagnosis has been made. The declining autoantibody titres over time has been attributed to diminished antigenic stimulation, due to progressive destruction of the adrenal cortex.[2] At least two studies have documented a decline of 21-hydroxylase autoantibodies and ACA with increasing duration.[49 50] A similar waning of autoimmunity occurs in coeliac disease where reduction of anti-TTG levels is observed after introducing a gluten-free diet.[51] Similarly, anti-Tg titres normalise once the thyroid is ablated for malignancy.[52] Finally, in T1DM, ICA prevalence declines with time after diagnosis, so that only 5 10% are ICA positive ten years after diagnosis.[15]

4.8 Conclusions

In summary, it has been shown, for the first time, that despite the high prevalence of tuberculosis and HIV in South Africa, autoimmune Addison's disease is by far the most common cause of adrenal insufficiency. HLA DQB*0201 and *0302 correlate with adrenal autoimmunity, while a novel finding of this study is that *0601 may be protective. Despite measurement long after diagnosis, markers of autoimmunity persist in many patients with Addison's disease. 21-hydroxylase autoantibodies identified twice as many autoimmune Addison's disease patients as ACA and therefore appear to be a more sensitive marker of an autoimmune aetiology in patients with long-standing disease.

It is important to highlight the fact that the cohort studied was predominantly of European ancestry (white), and it is difficult to predict whether the same distribution of causes for Addison's disease would be seen if the participants more closely reflected the demographic profile of the country, that is the majority being black African, as the black African group, although small, was negative for adrenal autoantibodies. Future studies should focus on identifying more patients with Addison's disease in this group, in order to address this question.

4.9 References

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Chapter 5
Lipids and lipoproteins, and markers of cardiovascular disease in primary hypoadrenalism

5.1 Introduction

For several decades, clinicians have asserted that the survival of patients with Addison's disease on replacement therapy has been similar to the background population. A Norwegian study corroborated the finding that the overall mortality rate was normal, but it found that the sub-group of males, younger than 40 years of age, suffered excess mortality due to adrenal failure, infection and sudden death.[1] A Swedish publication has also contested the original finding that the survival rate is similar to the background population. The study found that the relative risk of premature death was 2.19 and the risk of death, due to CVD from ischaemic heart and cerebrovascular disease, was doubled.[2] The reasons for this accelerated mortality were not investigated, but supra-physiological doses of GCs, resulting in dyslipidaemia and hypertension, could have contributed to this observed increased rate of mortality.[3] There are a number of both human and animal studies that indicate GCs potential for adversely affecting the lipid profiles. Elevated TC,[4-7] raised TG,[8 9] increased LDLC[5 6 10] and reduced HDLC[6] have been found in association with GCs. Thus, the accelerated mortality seen in Addison's disease may be due to adverse lipid profiles as a consequence of supra-physiological GC replacement. Therefore, it is clinically relevant to describe the lipid, lipoprotein profiles and markers of CV inflammation associated with Addison's disease, with a view to comparing these to healthy control subjects, as well as available normative data.

5.2 Aims

The purpose of this study is to establish whether patients with Addison's disease have an adverse lipid profile, raised markers of inflammation and higher CVD Framingham risk than controls.

The specific objectives of this study are to determine:

i. whether patients with Addison's disease have adverse lipid profiles
ii. if a correlation exists between doses of hydrocortisone replacement and the parameters used to evaluate lipid and lipoprotein metabolism
iii. whether markers of CVD inflammation are elevated in Addison's disease
iv. the Framingham CVD risk in patients with Addison's disease, compared with unaffected peers
v. how lipids and lipoprotein metabolism of any sub-group of South African Addison's patients compare with a Swedish Addison's sub-group that has been matched for age, gender, ethnicity and BMI.

5.3 Patients and methods

In this section the patients, healthy control subjects and assays for lipids, lipoproteins and biochemical markers of CVD, will be discussed.

5.3.1 Patients

The patients enrolled in the Addison's disease cohort, described in Chapter 3, were eligible for inclusion in this study. All participants were sufficiently well to receive ambulatory care, and the clinical information was obtained from a combination of the referring physicians and the participants in the study. The assumption was made that the patients complied with their chronic replacement medication, as verification of compliance could not be carried out. However, poor treatment compliance could be expected to some degree, as in another study,

non-adherence to taking chronic medication in CV patients varied between 31% and 58%.[11]

5.3.2 Matching with healthy control subjects

Healthy control subjects were recruited from volunteer blood donors. They were matched for ethnicity, BMI, age and gender with Addison's disease patients. As there were too few Asian subjects, and the black subjects were regarded as both too few and heterogeneous, comparison of the Addison's disease cohort with controls was limited to white and mixed ancestry subjects. Even within these sub-groups, matching was imperfect. As a generalisation, there was far more potential for choice in white healthy control subjects, compared to the other ethnic groups. Thus, white healthy control subjects could be selected from the blood donor clinic in a ratio of 1:10, while the mixed ancestry subjects were selected in a ratio of 1:4, indicating that for white healthy control subjects there were ten people to choose from for every one selected and in the case of mixed ancestry, there were four people to choose from to select one healthy control subject.

5.3.3 Matching with Swedish subjects

Fifty-seven Swedish patients with Addison's disease were matched with South African Addison's disease patients for age, gender, ethnicity and BMI.

5.3.4. Study methods

Demographic and clinical data were collected at enrolment. Sera were used to determine the TC, HDLC and TG. LDLC was calculated using the Friedewald equation, as shown below.

Additional approval was obtained from the University of Cape Town and from local research committees, where required, to conduct this study. The ethics review board of the University of Gothenburg approved the sub-study of matched South African and Swedish patients. This cohort was selected for chronic stable

Addison's disease.

5.3.5 Assays for lipids, lipoproteins and biochemical markers of CVD

The assays for TG, TC, NEFA and RBG were carried out with commercially available enzymatic kits, using standard curves and calibrators. The respective kits for TG, TC, NEFA and glucose were KAT triglycerides, category number T801, using a glycerol-3-phosphate oxidase method; KAT cholesterol, category number CH704, using cholesterol oxidase-phenol; peroxidase method, Roche free fatty acids category number 11383175001, using colorimetric assay; and KAT glucose, category number GP747, using a glucose oxidase method. The HDLC was performed according to the first step in the Gidez assay, which yields HDLC in the supernatant of a heparin-Mn precipitation of apoB-containing lipoproteins. LDL particle size measurement was performed, using non-denaturing gradient gel electrophoresis. The LDLC was calculated by the Friedewald equation, as shown below:[12]

LDLC = TC-HDLC-(TG/2.18), provided TG <4.5 mmol/L

The hs-CRP was performed using the high-sensitivity CRP immuno-turbidometric assay (Roche Diagnostics, GmbH, Mannheim, Tyskland), demonstrating a coefficient of variation (c.v.) of 4% and 3% at serum concentrations of 1 mg/L and 15 mg/L respectively. The Framingham risk was calculated using the algorithm to include diabetes so that comparisons could be made across the entire cohort.[13] Currently, diabetes is regarded as a secondary prevention equivalent for CVD.[14]

5.3.6 Statistical methods

Variables were tested for their distribution using the Shapiro-Wilk test. TC and LDLC were found to be normally distributed, whereas the remaining variables were non-normally distributed Assessment of statistical significance among non-parametric data was established using the Mann-Whitney test. The normally

distributed variables were compared using the t-test. Proportions were compared with Chi-squared tests and replaced by Fisher's exact test for small samples. Associations were determined using Spearman or Pearson regression analyses. The predictors for TG and HDLC were calculated using univariate and multivariate Cox hazards regression analyses. Significance was accepted when the *p*-value was <0.05.

5.4 Results

The results of this study are discussed below.

5.4.1 Description of the patients enrolled in the South African Addison's study

As seen in Figure 19, 146 of the 161 patients referred for enrolment in the South African Addison's study were included in the analysis of lipids, lipoproteins and markers of CVD.

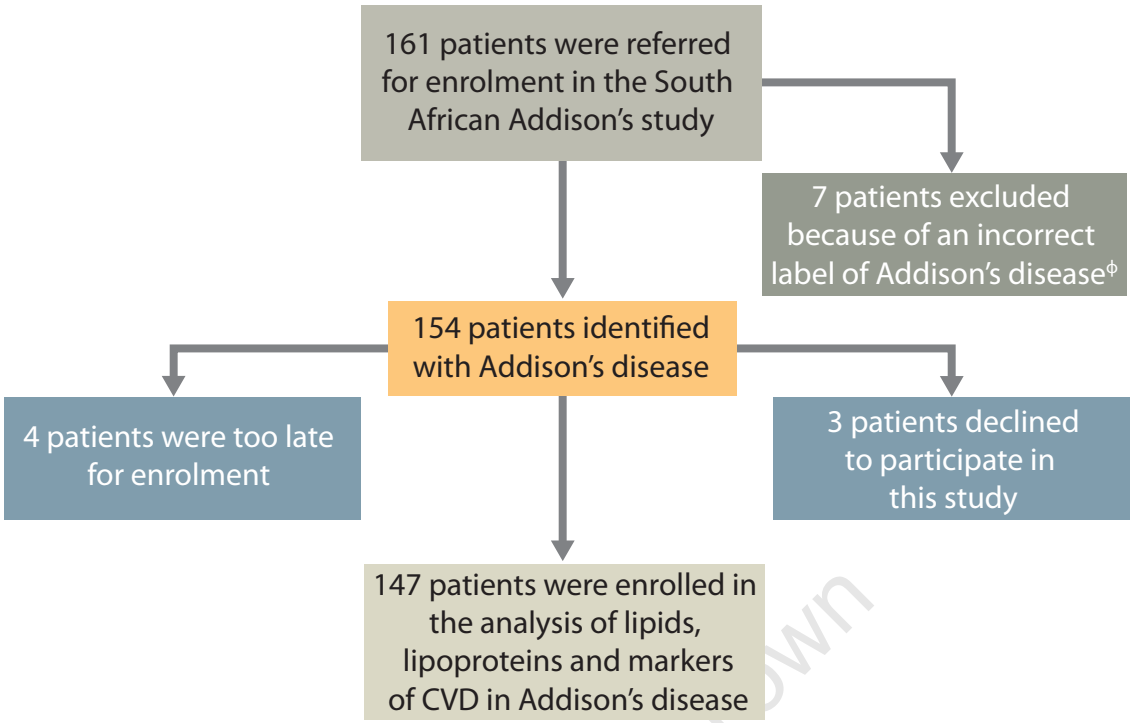

Figure 19: Flow diagram of South African Addison's patients enrolled in the lipids, lipoproteins and markers of cardiovascular disease study. $^{\Phi}$Seven patients were excluded because of an incorrect diagnosis, two had normal ACTH stimulation tests, two had secondary hypoadrenalism, one had bilateral adrenalectomy for Cushing's disease and one had suppression of the hypothalamic pituitary adrenal axis (HPA) axis related to previous steroid use for another indication.
CVD: Cardiovascular disease

The clinical characteristics of this cohort, defined as all patients, subjects who were neither diabetic nor on lipid-lowering therapy and diabetics not on lipid-lowering therapy are outlined in Table 20. The table depicts risk for myocardial infarction using an older risk-calculation algorithm, so that the absolute risk could be assigned to diabetic subjects. Recent guidelines classify diabetes as a secondary prevention equivalent and do not provide an absolute risk estimate for this latter group.[15] The proportions of the cohort with coexistent CV risk factors are also shown. The most prevalent CV risk factor in the whole group was hypertension (15%), followed by diabetes mellitus (14%). About 13% of the cohort was on lipid-lowering therapy and 8% were smokers.

In examining the sub-groups of Table 20 the whole cohort of Addison's patients included children. It explains the lower, but non-significant median age, compared with the other two groups, which comprised exclusively adults. As expected, there were more hypertensive patients in the diabetic sub-group on no lipid-lowering therapy compared to the sub-group without diabetes and also not on lipid-lowering therapy. There were no smokers in the diabetic sub-group, it is speculated that that these patients may have benefited from effective anti-smoking counselling. As expected, the median Framingham risk was greater in the diabetic subjects compared to the non-diabetics, with neither sub-group receiving lipid-lowering therapy. This was corroborated by a higher proportion of patients in the diabetic sub-group with a >20% Framingham risk of developing CVD in 10 years, compared to the non-diabetic sub-group. Nevertheless, the hs-CRP and the proportion of the cohort with predominant small dense LDL was no different between these two groups.

The patients on lipid-lowering therapy {(n = 19; median age (IQR) 57.0 (45.0-67.5) years} and diabetics {(n = 20, median age (IQR) 52.0 (39.0-59.0) years}, appeared older than the remainder of the cohort not using lipid-lowering therapy {(n = 127, median 44.0 (30.5-60.0) years} and that were non-diabetic {(n = 126, median 45.0 (31.5-61.0) years, but the differences were not significant: p = 0.69 and p = 0.42 respectively. There was a predominance of white and mixed ancestry patients in the sub-groups receiving lipid-lowering therapy and diabetic therapy, similar to the remaining cohort without these risk factors. The differential health-care in South Africa could explain the predominance of lipid-lowering treatment in white ancestry patients. Interestingly though, the ethnic distribution of patients receiving lipid-lowering therapy was not statistically different between the white and mixed ancestry patients. The increase in median age in the diabetic group, compared to non-diabetics (61 versus 48 years) not receiving lipid-lowering therapy was not significant. Among the diabetics, the ethnic make-up was different from the remainder of the cohort, as there were no black diabetics. There is a higher

risk of developing hypertension with increasing age, and the high prevalence of hypertension associated with diabetes is a well recognised CVD cluster.[16 17] There was a significantly greater proportion of diabetics (55%) who received lipid-lowering therapy than the remaining cohort (5.5%; p = 0.0001), reflecting the gravity of diabetes as a CVD risk factor. However, as this intervention was not uniformly implemented, it did not comply with recommendations that all diabetics should receive lipid-lowering therapy, apart from exceptional circumstances.

5.4.2 Cardiovascular risk

The Framingham 10-year risk was significantly higher in diabetics, compared to the remaining cohort, having excluded subjects on lipid-lowering therapy in both sub-groups (Table 20). Data from the Mamre study, a community-based study of CVD risk in people of mixed ancestry from Cape Town were used to estimate the Framingham risk as a comparison to risk in Addison's disease patients.[18] The CVD risk in the Mamre subjects was 14% for men and 8% for women in the same median age group of 46 years.[18] For the whole Addison's disease cohort, the risk was 12.1% and 14.5% for males and females respectively, which may be partly influenced by the younger median (IQR) ages of males, which was 37.0 (17.8-54.0) years, compared to females, which was 54.0 (39.5-63.0) years. By excluding patients in the Addison's disease cohort on lipid-lowering therapy and those with diabetes mellitus, the Framingham risk estimate of ≥20% for 10 years decreased from 36% to 32% (p = 0.01). The average risk is high enough to consider lipid-modifying treatment.

Table 20: Description of the Addison's disease patients: whole study population and sub-sets of non-diabetics and diabetics not receiving lipid-lowering therapy

	All patients	**Non-diabetic not on lipid-lowering therapy**	**Diabetics not on lipid-lowering therapy**	***p* - value** **Diabetic versus non-diabetic**
	N =146	*N* =117	*N* = 11	
Age in years (IQR)	46.0 (32-60.0)	48.0 (44.5-61.0)	61.0 (37.0-70.5)	0.11
‡Gender *N* (%)	90 (61)	71 (61)	7 (64)	0.89
Ethnicity *N* (%) White Mixed ancestry Asian Black	 96 (66) 34 (23) 5 (3) 11 (8)	 74 (69) 28 (24) 4 (3) 11 (10)	 7 (64) 4 (36) 0 (0) 0 (0)	0.57
Hypertension *N* (%)	22 (15)	7 (6)	4 (37)	**0.004***
Smokers *N* (%)	11 (8)	9 (8)	0(0)	0.74
Hydrocortisone dose mg (IQR)	25.0 (20.0 - 30.0)	25.0 (20.0 - 30.0)	20.0 (15 - 30.0)	0.32
Framingham CVD 10 - year risk (IQR) Ω	13.3 (5.3-25.4)	11.9 (5.24 - 21.5)	25.4 (17.5 - 35.0)	**0.002***
Proportion of cohort with Framingham risk ≥ 20% CVD for 10 years Ω *N* (%)	53 (36)	37 (32)	8 (70)	**0.02***
Proportion of cohort with small dense LDL *N* (%)	22 (15)	15 (13)	2 (18)	0.97
NEFA µmol/L (IQR)	345.0 (140.8 - 663.5)	329.0 (139.0 - 589.0)	371.0 (356.0 - 655.0)	1.0
hs-CRP mg/L (IQR)	2.2 (1.0 - 6.4)	2.85 (1.48 - 7.43)	3.3 (1.42 - 10.5)	0.83

Ω: Calculation of the absolute risk of myocardial infarction over 10 years in individuals without heart disease by combining several conventional risk factors in 87 non-diabetic on no lipid-lowering therapy subjects and 10 diabetics receiving no lipid-lowering therapy. Risk takes cognisance of

the presence of diabetes and is only estimated in individuals greater than 30 years of age. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation. 1998;97(18):1837-1847.[13]

N: Number

‡: Females

Median: Age, total daily hydrocortisone dose, Framingham CVD 10-year risk, hs-CRP

IQR: Interquartile range

CVD: Cardiovascular disease

LDL: Low density lipoprotein

hs-CRP: Highly sensitive C-reactive protein

$p < 0.05$ considered significant

$^{*}p < 0.05$

5.4.3 Descriptive lipid and lipoprotein data of South African Addison's disease patients

The mean ± SD TC for the entire cohort was 5.7 ± 1.55 mmol/L. As seen in Table 21, one subject had hypobetalipoproteinaemia (TC < 2.5 mmol/L) and 18 subjects (14%) had severe hypercholesterolaemia (TC >7.5 mmol/L). The possibility of a monogenetic disorder or secondary cause could not be clarified. Only 44 (35%) of the patients had a low risk TC of <5.0 mmol/L, and the remaining 63 patients (50%) had moderate hypercholesterolaemia (TC ranging from >5.0 mmol/L to <7.5 mmol/L).[19] The median and IQR TG level for the South African Addison's disease patients was 1.67 (1.10-2.62) mmol/L. Of all the patients, 39 (31%) had a TG of ≥2.3 mmol/L, which distinguishes a Fredrickson IIa from a Fredrickson IIb hyperlipidaemia. A TG of >1.7 mmol/L is not ideal, and 62 patients (49%) exceeded this threshold.[20] Fredrickson type IV hyperlipidaemia, inferred by hypertriglyceridaemia (TG >5.0 mmol/L), was present in four patients. Agarose electrophoresis was not performed to distinguish between type IIb and type IV hyperlipidaemia, so that the remaining cohort of TG 2.3-5.0 mmol/L remains uncharacterised. There were 35 subjects (28%) who had a TG concentration between 2.3 mmol/L and 5.0 mmol/L, who potentially may have had either

type IIb or type IV hyperlipidaemia.[21] Four patients had hypertriglyceridaemia, meriting referral to a specialist lipid clinic and who may require medication other than 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCo-A-reductase) inhibitors. No patients had severe hypertriglyceridaemia (>15 mmol/L) and thus overall, the risk for pancreatitis from hypertriglyceridaemia was low.

The median plasma HDLC and IQR was 0.78 (0.52-1.08) mmol/L. The levels of HDLC ranged from 0.03-2.87 mmol/L. Hypoalphalipoproteinaemia, considered a CVD risk factor when the concentration is <1.0 mmol/L, was identified in 82 patients (73%). Ten patients (9%) were identified as having extremely low HDLC with levels of <0.25 mmol/L. Theoretically, this may be compatible with genetic disorders of HDL metabolism or a severe acute phase response.[22] However, when comparing male patients with their controls, and similarly, female patients with their controls, no difference in HDLC was found. Consideration needs to be given to prolonged storage of samples as a possible explanation for the unexpectedly low levels of HDLC.

The mean LDLC was 4.1 mmol/L, but hypobetalipoproteinaemia (LDLC <1.5 mmol/L) was identified in only one patient. The ideal LDLC concentration of between 1.5 mmol/L and 2.5 mmol/L was present in 12 patients (11%). There were 14 patients (13%) that had an LDLC of between 2.5 mmol/L and 3 mmol/L, representing a low, but not a negligible risk of developing CVD, depending on the clinical context. There were 55 patients (51%) that had an LDLC of between 3.0 mmol/L and 5.0 mmol/L, and 25 patients (23%) were deemed to have high risk LDLC (>5 mmol/L).[20]

Table 21 also shows the descriptive lipid and lipoprotein data for the sub-group with diabetes compared with the non-diabetic sub-group. The proportion with diabetes who had a TG of >2.3 ≤ 5.0 mmol/L and LDLC of >5.0 mmol/L was greater than the non-diabetic patients (35% versus 26%; $p = 0.04$ and 35% versus 21%;

p = 0.0006 respectively). Overall, diabetes was associated with higher LDLC levels and moderately raised TG, potentially in the same individuals. Interestingly, severely raised TG was present in some non-diabetic patients with Addison's disease. Moderate hypercholesterolaemia and moderate LDL were slightly less prevalent in the diabetic cohort compared to the non-diabetic cohort; however severe LDL hypercholesterolaemia was more prevalent in the diabetic sub-group.

Table 21: Descriptive lipid and lipoprotein data of South African Addison's disease patients, arbitrarily defined cut-off points for significant dyslipidaemia

TG mmol/L		<1.7	1.7-2.3	>2.3 ≤ 5.0	>5.0
Non-diabetic cohort Diabetic cohort Whole cohort Missing data *N* (%) Diabetic versus non-diabetic *p*-value	*N* = 106 (%) *N* = 20 (%) *N* = 126 (%) *N* = 20 (14)	54 (51) 10 (50) 64 (51) 0.84	20 (19) 3 (15) 23 (18) 0.31	28 (26) 7 (35) 35 (28) **0.04***	4 (4) 0 (0) 4 (3) 1.0
TC mmol/L		<2.5	≥ 2.5-5.0	> 5.0 ≤ 7.5	> 7.5
Non-diabetic cohort Diabetic cohort Whole cohort Missing data *N* (%) Diabetic versus non-diabetic *p*-value	*N* = 106 (%) *N* = 20 (%) *N* = 126 (%) *N* = 20 (14)	0 (0) 1 (5) 1 (1) 0.16	37 (35) 7 (35) 44 (35) 1.0	57 (54) 8 (40) 63 (50) **0.04***	12 (11) 4 (20) 18 (14) 1.0
HDLC mmol/L		≤ 0.25	> 0.25 ≤ 1.0	> 1.0	
Non-diabetic cohort Diabetic cohort Whole cohort Missing data *N* (%) Diabetic versus non-diabetic *p*-value	*N* = 94 (%) *N* = 18 (%) *N* = 112 (%) *N* = 34 (23)	7 (7) 1 (5) 10 (9) 0.4	63 (67) 12 (67) 72 (64) 1.0	24 (26) 5 (28) 30 (27) 0.65	
LDLC mmol/L		<1.5	≥ 1.5-2.5	> 2.5 ≤ 3	> 3.0-5.0	>5.0
Non-diabetic cohort Diabetic cohort Whole cohort Missing data *N* (%) Diabetic versus non-diabetic *p*-value	*N* = 90 (%) *N* = 17 (%) *N* = 107 (%) *N* = 39 (27)	0 (0) 1 (6) 1 (1) 	10 (11) 2 (12) 12 (11) 0.75	12 (13) 2 (12) 14 (13) 0.77	49 (54) 6 (35) 55 (51) **0.0001***	19 (21) 6 (35) 25 (23) **0.0006***

N: Number
TG: Triglyceride
TC: Total cholesterol
HDLC: High density lipoprotein cholesterol
LDLC: Low density lipoprotein cholesterol
$p < 0.05$ considered significant
$*p < 0.05$

Among Addison's patients, the sub-group of treated diabetics, all on oral hypoglycaemic agents or insulin or both, had similar concentrations of plasma NEFA as non-diabetics ($p = 0.74$). It is not clear whether the diabetic treatment counteracted the usual diabetes-induced rise of NEFA, or whether Addison's disease per se or its treatment, blunts the expected rise in plasma NEFA concentrations. Other possible relationships and plasma NEFA concentrations were explored (Table 22). In the sub-group of diabetic Addison's patients, NEFA was not correlated with any of the plasma lipids or lipoproteins (Spearman correlations for TC and LDLC; Pearson correlations for TG and HDLC). It was surprising that NEFA did not correlate with the concentration of TG in either the diabetic or the non-diabetic groups. However, in the whole cohort, NEFA was positively associated with TC and LDLC, and there was a trend to a positive correlation with HDLC, in the non-diabetic sub-group, but not in the diabetic sub-group. It is difficult to explain the observation that NEFA was positively correlated with TC and LDLC. It is assumed that preservation of samples is not involved in this positive association and that plasma contains no active lipoprotein lipase to hydrolyse TG. However, plasma may have some residual hepatic lipase or cholesterol esterase activity, which could have influenced the concentrations of TC or LDLC.[23]

Table 22: The correlations of plasma NEFA in Addison's disease patients

	Addison's patients whole cohort		**Non-diabetic**		**Diabetic**	
TG	*r* = -0.108	*p* = 0.25	*r* = - 0.162	*p* = 0.11	*r* = 0.296	*p* = 0.23
TC	*r* = 0.252	*p* = **0.006***	*r* = 0.287	*p* = **0.004***	*r* = 0.049	*p* = 0.85
HDLC	*r* = 0.187	*p* = 0.06	*r* = 0.267	*p* = **0.01***	*r* = -0.197	*p* = 0.46
LDLC	*r* = 0.255	*p* = **0.01***	*r* = 0.270	*p* = **0.01***	*r* = 0.096	*p* = 0.73

TG Triglyceride

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low density lipoprotein cholesterol

r: Correlation coefficient

$p < 0.05$ considered significant

*$p < 0.05$

In order to establish whether the lipid profiles were different in diabetic patients compared to non-diabetic patients, subjects not using lipid-lowering therapy were compared (Table 23). Neither the TC, LDLC, TG, NEFA, random blood glucose (RBG) concentrations nor the proportion of small dense LDL was different between the two groups.

Table 23: Comparison of lipid profiles of diabetics with non-diabetics not using lipid modifying treatment

	Diabetic	Non-diabetic	*p*-value Diabetic versus non-diabetic
Number	11	117	
TG mmol/L (IQR)	1.57 (1.08 - 2.57) (*N* = 9)	1.65 (1.09 - 2.62) (*N* = 97)	0.75
TC mmol/L (SD)	5.30 (1.62) (*N* = 11)	5.62 (1.50) (*N* = 97)	0.53
HDLC mmol/L (IQR)	0.77 (0.73 - 0.86) (*N* = 11)	0.78 (0.50 - 1.02) (*N* = 86)	1.0
LDLC mmol/L (SD)	3.72 (1.53) (*N* = 9)	4.04 (1.33) (*N* = 82)	0.57
Proportion with small dense LDL *n/N*(%)	2/ 11 (18)	12/ 97 (12)	0.59
NEFA µmol/L (IQR)	371.0 (209.0 - 655.5) (*N* = 11)	308 (139.0 - 584.3) (*N* = 90)	0.26
RBG mmol/L (IQR)	5.48 (4.87 - 12.13) (*N* = 11)	5.3 (4.75 - 6.15) (*N* = 97)	0.22

Median: TG, HDLC, NEFA and RBG

Mean: TC and LDLC

TG: Triglyceride

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low density lipoprotein cholesterol

NEFA: Non-esterified fatty acids

RBG: Random blood glucose

IQR : Interquartile range

N: Number of patients with either diabetes or not diabetic

SD: Standard deviation

$p < 0.05$ considered significant

The underlying two conditions least likely to induce derangements in lipids were deemed to be ALD and isolated autoimmune Addison's disease. So, in order to assess whether the aetiology of Addison's disease could be contributing to lipid abnormalities, these two conditions were compared to the remaining causes for Addison's disease. The TG concentrations were found to be significantly lower among patients with ALD and isolated autoimmune Addison's disease, compared to the remaining cohort. The small difference in TG is, however, not useful at the clinical level to discern the different causes of Addison's disease.

5.4.4 Lipid and lipoprotein data in white and mixed ancestry patients compared to their respective controls

This analysis was performed on the cohort of white and mixed ancestry patients over the age of 30 years undergoing the Framingham risk calculation, irrespective of intervention for dyslipidaemia. As seen in Table 24, the median ages and BMI confirm adequate matching between the patients and their respective controls. In both categories, the mean TC and LDLC did not differ between the patient and control groups. However, the median TG levels were significantly higher in the mixed ancestry patients versus the mixed ancestry controls, despite the former having a lower median BMI.[18] There were no individuals with extreme TG deviations in either group. The explanation for this could lie in the elevation of TG by the cause(s) of Addison's disease or its treatment. The median HDLC was lower in both the white patients and the patients of mixed ancestry versus their respective controls. The hs-CRP was increased compared to their respective controls. There was a significantly greater proportion of small dense LDL among the white and mixed ancestry patients versus their respective controls.

The NEFA levels were lower in the white patients compared to their controls, and although the levels were lower in the mixed ancestry patients compared to their controls, it was only of borderline significance. Due to their extreme values, the two highest outlying values of NEFA were removed from the mixed ancestry

Addison's patients. There was no difference in NEFA between the mixed ancestry patients and the control group ($p = 0.05$), although the trend remained. The median RBG concentrations were surprisingly higher among the white controls versus the white patients (5.93 mmol/L versus 5.36 mmol/L; $p = 0.0001$). The mixed ancestry patients also had a lower median RBG compared with their respective controls (5.7 mmol/L versus 5.9 mmol/L; $p = 0.005$).

Overall, a greater proportion of the Addison's patients had small dense LDL particles, compared with the controls. The median TSH levels did not differ between the white {(1.51, IQR (0.57-2.00)} mIU/L and mixed ancestry {(1.16, IQR (0.85-1.80)} mIU/L patients ($p = 0.17$). As expected, the RBG was significantly greater in the white patients with diabetes compared to the white patients without. The impact of diabetes mellitus was also analysed in white and mixed ancestry patients, compared to their respective controls. These results are summarised in Table 25. Diabetes mellitus in the mixed ancestry patients was associated with a greater LDLC concentration (4.89 mmol/L versus 3.66 mmol/L $p = 0.04$). Although inequalities exist in health care in South Africa, where whites in the private sector may have more access to lipid-modifying therapy than other groups in public health-care systems, diabetic white patients received lipid-lowering therapy as frequently as the mixed ancestry patients ($p = 0.4$).

Table 24: Comparisons of lipid profiles and atherosclerotic risk in the cohort of patients and their selected controls, undergoing the Framingham risk calculation

Variable	White patients	White controls	p_1-value White patients versus white controls	Mixed ancestry patients	Mixed ancestry controls	p_2-value Mixed ancestry patients versus mixed ancestry controls
Age in years (IQR)	43.0 (31.0-59.0) *N* = 81	46.0 (32.0-54.0) *N* = 96	0.949	52.0 (32.8-65.8) *N* = 32	41.0 (35.0-49.0) *N* = 34	0.1
BMI kg/m^2 (IQR)	24.3 (21.5-28.1) *N* = 71	25.9 (22.0-30.0) *N* = 96	0.08	27.5 (22.5-31.8) *N* =22	31.2 (24.3-32.5) *N* = 22	0.4
TG mmol/L (IQR)	1.72 (1.02-2.36) *N* = 71	1.43 (0.92-2.11) *N* = 96	0.23	1.72 (1.32-2.51) *N* = 28	1.2 (0.94-2.1) *N* = 34	**0.04***
TC mmol/L (SD)	5.69 (1.51) *N* = 71	5.9 (1.13) *N* = 96	0.36	5.45 (1.44) *N*= 28	5.4 (1.41) *N*= 34	0.96
HDLC mmol/L (IQR)	0.77 (0.57-1.00) *N* = 63	1.12 (0.99-1.38) *N* = 62	**0.0001***	0.77 (0.44-0.92) *N* = 25	0.94 (0.8 4-1.11) *N* = 28	**0.01***
LDLC mmol/L (SD)	4.08 (1.40) *N* = 60	3.87 (0.99) *N* = 62	0.34	3.87 (1.2) *N* = 24	3.74 (1.4) *N* = 28	0.71
Proportion small dense LDL *n/N* (%)	11/81 (14)	3/ 96 (3)	**0.02***	5/30 (17)	0/33 (0)	**0.04***
NEFA µmol/L (IQR)	349.0 (185.0-612.0) *N* = 69	483 (348.5-660.0) *N* = 92	**0.005***	220.5 (72.8-569.0) *N* = 26	402.5 (265.5-614.8) *N* = 33	0.05
RBG mmol/L (IQR)	5.36 (4.55-6.15) *N* = 70	5.93 (5.62-6.4) *N* = 92	**0.0001***	5.7 (4.97-6.35) *N* = 33	5.88 (5.64-6.46) *N* = 32	**0.005***
hs-CRP mg/L (IQR)	2.3 (0.90-5.03) *N* = 80	1.5 (0.59-2.55) *N* = 96	**0.01***	6.4 (1.75-12.5) *N* = 32	1.80 (1.2-3.6) *N* = 33	**0.01***

Variable	White patients	White controls	p_1-value White patients versus white controls	Mixed ancestry patients	Mixed ancestry controls	p_2 -value Mixed ancestry patients versus mixed ancestry controls
TSH$^{\Phi}$ mIU/L (IQR)	1.51 (0.57-2.00)			1.16 (0.85-1.80)		

Median: Age, BMI, TG, HDLC, NEFA, RBG, hs-CRP and TSH

Mean: TC and LDLC

BMI: Body mass index

TG: Triglyceride

HDLC: High density lipoprotein cholesterol

LDLC: Low density lipoprotein cholesterol

NEFA: Non-esterified fatty acids

RBG: Random blood glucose

hs-CRP: Highly sensitive C-reactive protein

TSH: Thyroid stimulating hormone

IQR: Interquartile range

N: Number

SD: Standard deviation

p_1*:* Comparison between white patients and white controls

p_2*:* Comparison between mixed ancestry patients and mixed ancestry controls

$p < 0.05$ considered significant

$^{*}p < 0.05$

Φ: TSH was only measured in patients and not controls

5.4.5 The effect of diabetes on lipid and lipoprotein data in this cohort

There were no differences in any of the lipids and lipoproteins between the South African patients with Addison's disease, with or without diabetes. As expected, the median RBG (IQR) was higher in the sub-group with diabetes mellitus (9.83 mmol/L, range 5.86-12.58 mmol/L versus 5.27 mmol/L, range 4.74-6.30 mmol/L; $p = 0.0001$). In the sub-group of diabetics Addison's patients not on lipid-lowering

therapy compared with those diabetic patients on lipid-lowering therapy the median TG and IQR was {1.51 (0.90-2.6) mmol/L versus 1.83 (1.3-2.6) mmol/L; p = 0.36}, the mean (SD) {TC 5.30 (1.54) mmol/L versus 6.57 (2.14) mmol/L; p = 0.17}, median and IQR HDLC was {0.79 (0.73-1.03) mmol/L versus 0.65 (0.4-1.6) mmol/L; p = 0.64} and mean (SD) LDLC was {3.72 (1.53) mmol/L versus 4.81 (1.67) mmol/L ; p = 0.19}. The NEFA did not differ in these two groups; p = 1.0. Overall there were no differences in the lipid and lipoprotein concentrations between the diabetic sub-groups on lipid-lowering therapy and those without. However, values before and after initiating lipid-lowering therapy were not available. These findings may be due to confusion caused by the lipid-lowering treatment, as there is an inherent bias towards treating diabetic patients with lipid-lowering therapy because it is a secondary prevention equivalent. Diabetics tended to have moderately raised TG levels and both moderate and severe LDLC elevation (Table 21). A mildly elevated TG level is expected with diabetes, along with lower HDLC, but this is not seen in this study, possibly because of the small numbers and other influences on lipid profiles. The reason for the elevated LDLC is not obvious, but it may be influenced by diet, genetic predisposition and renal disease.

Table 25: The influence of diabetes on lipid, lipoproteins and other data in white and mixed ancestry patients

Variable	White patients with diabetes	White patients without diabetes	p_1 - value White patients with diabetes versus white patients without	Mixed ancestry patients with diabetes	Mixed ancestry without diabetes	p_2 - value Mixed ancestry patients with diabetes versus mixed ancestry patients without
Number	*N* = 13	*N* = 83		*N* = 7	*N* = 27	
Age in years (IQR)	46.0 (38.0-55.0)	44.0 (31.8-59.3)	0.98	64.0 (51.0-72.0)	59.0 (29.0-72.0)	0.23
BMI kg/m^2 (IQR)	24.7 (22.6-27.1)	24.7 (21.7-29.3)	0.65	30.7 (29.7-31.6)	27.4 (22.5-30.9)	0.37
TG mmol/L (IQR)	1.45 (1.18-2.14)	1.62 (1.07-2.37)	0.65	2.31 (1.91-2.87)	1.69 (1.34-2.32)	0.48
TC mmol/L (SD)	5.62 (2.21)	5.87 (1.47)	0.7	6.36 (1.18)	5.28 (1.38)	0.11
HDLC mmol/L (IQR)	0.75 (0.6-1.2)	0.8 (0.6-1.0)	0.74	0.55 (0.3-0.88)	0.8 (0.45-1.0)	0.53
LDLC mmol/L (SD)	3.97 (1.87)	4.19 (1.33)	0.69	4.89 (0.79)	3.66 (1.13)	**0.04***
Proportion small dense LDL (%)	2/13 (15)	9/73 (12)	0.89	1/5 (20)	4/25 (16)	0.67
NEFA µmol/L (IQR)	356.0 (85-539)	352.0 (181-694)	0.55	612.0 (211.0-875.0)	569.0 (66.0-199.5)	0.17
RBG mmol/L (IQR)	8.65 (5.09-1.93)	5.26 (4.68-6.04)	**0.03***	9.83 (4.48-11.4)	5.58 (4.92-6.3)	1.0
hs-CRP mg/ml (IQR)	1.6 (0.8-3.2)	2.25 (0.95-5.05)	0.4	5.6 (3.4-12.0)	6.1 (0. 8-11.0)	0.60
TSH mIU/L (IQR)	0.94 (0.47-1.93)	1.56 (0.77-2.17)	0.26	1.50 (1.14-1.65)	1.14 (0.83-1.88)	0.61

Median: Age, BMI, TG, HDLC, LDLC, NEFA, RBG, hs-CRP and TSH

Mean: TC and LDLC

BMI: Body mass index

TG: Triglyceride
TC: Total cholesterol
HDLC: High density lipoprotein cholesterol
LDLC: Low density lipoprotein cholesterol
NEFA: Non-esterified fatty acids
RBG: Random blood glucose
hs-CRP: Highly sensitive C-reactive protein
TSH: Thyroid stimulating hormone
IQR: Interquartile range
SD: Standard deviation
p_1: Comparison between white patients and white controls
p_2: Comparison between mixed ancestry patients and mixed ancestry controls
$p < 0.05$ considered significant
$*p < 0.05$

5.4.6. General applicability of white and mixed ancestry control data

The white control subjects were compared to the white community study (CORIS) and the mixed ancestry control subjects were compared to the mixed ancestry community study (Mamre).[18 24] The mixed ancestry control subjects differed from the community study, in that they had higher TC, lower HDLC and higher LDLC levels. The proportion of subjects with high TG was greater in the mixed ancestry control subjects compared to the mixed ancestry community study. This may be due to considerably higher BMI levels in the mixed ancestry control subjects (31.2 kg/m^2) compared to those in the Mamre community study (24.5 kg/m^2). Therefore, the lipid and lipoprotein data from the mixed ancestry controls may not be applied to all the mixed ancestry in South Africa, while the data from the white controls can be applied to all the whites.

5.4.7 The description of lipid and lipoprotein data among black and Asian patients enrolled in the South African Addison's study

As there were few Asian and black patients among the South African Addison's disease patients ($n = 5$ and $n = 11$ respectively), they were not matched to controls (Table 26). The mean TC of black patients with Addison's disease appears to agree with the two separate community studies of black South Africans.[25 26] The TG and HDLC levels among Asian patients were strikingly abnormal, with the median TG and HDLC being 2.83 mmol/L and 0.66 mmol/L respectively. All of the Asian patients had LDLC of >3.0 mmol/L, TG of >1.7 mmol/L and HDLC of <1.0 mmol/L, and 75% had TC of >5 mmol/L. While the Asian sub-group of Addison's patients was very small, a significant proportion had an adverse lipid profile, unlike the black patient cohort, in which 33-50% had abnormal lipid parameters.

Formal statistical comparison with other ethnic groups is not of value because of the small numbers of Asian and black subjects. However, on inspection, the Asian sub-group was older than the white and mixed ancestry subjects, while the black sub-group were younger than both the white and mixed ancestry patients. The Asian patients appeared thinner than the white and mixed ancestry patients; while by comparison, the black patients were extremely lean. TG was elevated in Asian patients, compared to the black patients, but the TG in black patients was similar to both white and mixed ancestry patients. The TC in Asian patients appeared greater than in white patients, but similar to the mixed ancestry patients, whereas the TC of black patients was considerably lower than both the white and mixed ancestry patients. The HDLC of Asian patients was lower than that of both the white and mixed ancestry patients. The HDLC found in black patients appeared to be greater than the other ethnic sub-groups. LDLC was found to be higher in the Asian patients compared to white and mixed ancestry patients. Although there were only a few black patients, they exhibited much lower LDLC concentrations than the other ethnic sub-groups. While no Asian patients with small dense LDL were observed, the proportion of black patients who demonstrated small dense

LDL was similar to both the white and mixed ancestry patients.

The RBG of Asian patients was higher than the sub-groups of the white and mixed ancestry patients, even though none of the Asians was known to be diabetic, but the black patients exhibited similar RBG to the white and mixed ancestry patients. The hs-CRP appeared to be markedly elevated in the Asian sub-group compared to the other sub-groups, while the black sub-group demonstrated the lowest concentration of this CV inflammatory marker compared to the remaining ethnic sub-groups.

Table 26: Description of lipid and lipoprotein data in the Asian and black sub-groups with Addison's disease

Variable	‡Asian	‡Black	Clinical Parameter	Asian *n/N* (%)	Black *n/N* (%)
Age in years (IQR)	58.0 (50.0-67.0) *N* = 5	37.0 (6.37-68.5) *N* = 11		-	-
BMI kg/m^2 (IQR)	23.9 (23.4-27.6) *N* = 3	15.6 17.8$^{\sigma}$ *N* = 2		-	-
TG mmol/L (IQR)	2.83 (2.18-4.40) *N* = 4	1.88 (1.1-3.86) *N* = 6	‡TG ≥ 1.7 mmol/L	4/4 (100)	3/6 (50)
TC mmol/L (SD)	6.56 (1.5) *N* = 5	4.46 (1.00) *N* = 6	‡TC ≥ 5 mmol/L	3/4 (75)	2/6 (33)
HDLC mmol/L (IQR)	0.66 (0.50-0.66) *N* = 4	1.02 (0.80-1.23) *N* = 4	‡HDL <1.0 mmol/L	4/4 (100)	2/4 (50)
LDLC mmol/L (SD)	5.84 (0.11) *N* = 3	2.74 (0.99) *N* = 4	‡LDLC ≥ 3 mmol/L	3/3 (100)	2/4 (50)
Proportion with small dense LDL *n/N* (%)	0/4 (0)	1/6 (17)		-	-
NEFA µmol/L (IQR)	196.0 (139.0-5.0) *N* = 3	257.0 (179.0-75.0) *N* = 5		-	-
RBG mmol/L (IQR)	6.48 (5.93-8.42) *N* = 4	5.72 (5.12-6.83) *N* = 6	‡RBG ≥ 11.1 mmol/L	0/4 (0)	0/6 (0)
hs-CRP mg/L (IQR)	13.0 (2. 00-15.7) *N* = 4	1.50 (1.30-3.3) *N* =11	‡hs-CRP > 4.5 mg/L	2/5 (40)	2/6 (33)
TSH mIU/L (IQR)	1.81 (1.00-3.01) *N* = 5	1.94 (0.91-3.12) *N* = 10		-	-

Median: Age, BMI, TG, HDLC, LDLC, NEFA, RBG, hs-CRP and TSH

Mean: TC and LDLC

BMI: Body mass index

TG: Triglyceride

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low density lipoprotein cholesterol

NEFA: Non-esterified fatty acids
RBG: Random blood glucose
hs-CRP: Highly sensitive C-reactive protein
TSH: Thyroid stimulating hormone
‡: Numbers too low to be matched with controls
Freq: Frequency
IQR: Interquartile range
SD: Standard deviation
n: Total number of patients failing to achieve lipid targets
N: Total number of patients in these two ethnic groups
σ: Numbers too few to calculate median, therefore individual values are provided

5.4.8 Comparisons between patients and controls, according to the targets of the NCEP ATP III criteria

As shown in Table 27 the proportions of patients not achieving TG, TC, LDLC targets, according to NCEP ATP III guidelines,[20] were no different in white and mixed ancestry patients compared to their respective controls. The proportions of patients whose HDLC was <1.0 mmol/L were significantly higher in white patients compared to their respective controls only, while greater proportions of the mixed ancestry patients had abnormal hs-CRP levels, compared to their respective controls.

As mixed ancestry patients were previously disadvantaged and may not have enjoyed the same level of health-care as white patients, the extent to which these ethnic groups differed in failing to achieve NCEP ATP III targets were compared and no differences were noted in control of TG, TC, HDLC and RBG. The proportion with an abnormal hs-CRP was greater in the mixed ancestry patients, compared with the white patients.

Table 27: Comparisons of the proportion of patients and their respective controls, according to the cutpoints of the NCEP ATP III recommendations,[20] as well as RBG and hs-CRP

Variable	Proportion of white patients *n/N* (%) versus white controls *n/N* (%)	Proportion of mixed ancestry patients *n/N* (%) versus mixed ancestry controls *n/N* (%)
TG > 1.7 mmol/L	41/87 (47) vs. 34/97 (36) *p* = 0.1	16/30 (53) vs. 10/32 (31) *p* = 0.1
TC > 5 mmol/L	59 /86 (69) vs. 81/ 97 (84) *p* = 0.43	19/30 (63) vs. 18/34 (53) *p* = 0.56
HDLC < 1.0 mmol/L	56/77 (72) vs. a 18/63 (29) *p* = **0.0001***	20/26 (77) vs. 16/28 (57) *p* = 0.21
LDLC > 3 mmol/L	55/74 (74) vs. 52/63 (83) *p* = 0.34	21/26 (81) vs. 20/28 (71) *p* = 0.23
RBG > 11.1 mmol/L	6/85 (7) vs. 1/96 (1) *p* = 0.08	4/30 (13) vs. 0/33 (0) *p* = 0.09
hs-CRP > 4.5 mg/L	25/97 (26) vs. 15/97 (15) *p* = 0.11	19/34 (56) vs. 6/34 (18) *p* = **0.002***

TG: Triglyceride

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low-density lipoprotein cholesterol

RBG: Random blood glucose

hs-CRP: Highly sensitive C-reactive protein

vs.: versus

$p < 0.05$ considered significant

$*p < 0.05$

NCEP: National Cholesterol Education Programme

ATP III: Adult Treatment Panel III

n: Total number of patients who failed to achieve recommended lipid targets

N: Total number of patients and controls in these two ethnic groups

5.4.9 Patients using lipid-lowering therapy failing to achieve NCEP ATP III targets

Bias was likely because the patients using lipid-lowering therapy also received their medical care through private, rather than state facilities. 88% of the patients on lipid-lowering therapy had persistent levels of TC >5.0 mmol/L and 81% had persistent LDLC of >3.0 mmol/L, indicating that treatment for CV risk was not aggressively undertaken or that treatment compliance was incomplete (Table 28). Achievement of TG and LDLC targets did not differ in patients using or not using lipid-lowering therapy. On the other hand the proportion of patients who failed to achieve target TC was greater in those on lipid-lowering therapy than those patients not on this treatment. Patients not on lipid-lowering therapy had a higher proportion whose HDLC was <1.0 mmol/L. All patients not on lipid-lowering therapy should be assessed for their global CV risk because adverse lipid profiles are highly prevalent in this disease group. It is speculated that the lipid-lowering therapy may have been initiated in patients with poorer baseline lipid concentrations, than those who were not on this medication.

Table 28: Comparison of the lipid data of patients using lipid-lowering therapy versus those patients not on lipid-lowering therapy, using NCEP ATP III guideline criteria[20]

Variable	Lipid - lowering therapy *n/N* (%)	Not on lipid - lowering therapy *n/N* (%)	*p* - value
TG >1.7 mmol/L	12/18 (67)	50/108 (46)	0.17
TC > 5.0 mmol/L	16/18 (88)	68/108 (63)	**0.03***
HDLC < 1.0 mmol/L	7/17 (41)	71/95 (75)	**0.006***
LDLC > 3.0 mmol/L	13/16 (81)	67/91 (74)	0.11

TG: Triglycerides

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low-density lipoprotein cholesterol

NCEP: National Cholesterol Education Programme

ATP III: Adult Treatment Panel III

n: Total number of patients failing to achieve lipid targets

N: Total number of patients with available data either on or not on lipid-lowering therapy

$p < 0.05$ considered significant

$*p < 0.05$

5.4.10 The relationship between hydrocortisone and lipid fractions and other markers of cardiovascular risk

The relationship between the hydrocortisone dose, lipid fractions and other parameters of CV risk was assessed by linear regression analyses. As has been previously demonstrated in the literature, HDLC is also positively correlated with hydrocortisone in this study ($r = 0.3$; $p = 0.003$). Glucose is also positively correlated with hydrocortisone dose ($r = 0.32$; $p = 0.001$), but TSH is negatively correlated with hydrocortisone dose ($r = -0.20$; $p = 0.03$). These relationships of total daily hydrocortisone dose with HDLC, RBG and TSH are expected physiologically. When the dose of hydrocortisone was corrected for body weight, TG ($r = -0.24$; $p = 0.01$) and hs-CRP ($r = -0.3$; $p = 0.001$) were negatively associated for hydrocortisone, while HDLC remained positively associated, ($r = 0.38$; $p = 0.0001$).

5.5 Comparison of the sub-group of South African Addison's disease patients matched with the Swedish cohort

As seen in Table 29, the sub-group of South African Addison's disease patients, who were matched for ethnicity, gender, age and BMI with the Swedish Addison's patients, demonstrated an adverse lipid profile with lower doses of hydrocortisone. When patients on lipid-lowering therapy were excluded, TC, LDLC and HDLC remained different, while TG did not. The other CVD risk factors were however, similar in both cohorts. Although quality control should ensure comparability of data, the laboratories were not controlled for comparability of these results and

this could influence the findings. The implication is that South African Addison's disease subjects could have a much higher CV risk, as each of the lipid and lipoprotein parameters is far worse. The general applicability of this comparison to the whole study of matched Swedish and South African patients was assessed between the South African white patients who were included and those who were excluded. Although only age differed significantly, the general findings are likely applicable.

Table 29: Comparison of the sub-group of South African Addison's patients, matched with the Swedish cohort

	South African Cohort Mean[Ψ] *N*=57	SD	**Swedish Cohort** Mean[Ψ] *N*=57	SD	***p*-value[α] Swedish versus matched South African Addison's patients**
Age (years)	53.1	14.5	53.1	13.3	0.97
BMI (kg/m^2)	25.8	4.1	25.6	3.6	0.72
TG (mmol/L)	1.9	1.1	1.3	0.8	**0.002***
TC (mmol/L)	6.0	1.5	5.2	0.9	**0.001***
HDLC (mmol/L)	0.8	0.4	1.9	0.5	**0.001***
LDLC (mmol/L)	4.4	1.4	2.7	0.8	**0.001***
Total daily hydrocortisone dose (mg)	24.5	7.9	32.8	8.0	**0.001***
Total daily fludrocortisone dose (mg)	0.08	0.05	0.08	0.04	0.59
Disease duration (years)	14.8	11.6	17.8	11.7	0.17
Smoking *N* (%)	5 (9)		4 (7)		0.72
Hypertension *N* (%)	11 (19)		11 (19)		0.1
Diabetes *N* (%)	12 (21)		10 (18)		0.64
Lipid-lowering therapy *N* (%)	11 (19)		10 (18)		0.81

N = 57

N: Number

SD: Standard deviation

BMI: Body mass index

TG: Triglyceride

TC: Total cholesterol

HDLC: High density lipoprotein cholesterol

LDLC: Low-density lipoprotein cholesterol

α: Swedish patients were matched with South African patients by age, gender, ethnicity and BMI

$p < 0.05$ considered significant

$*p < 0.05$

Ψ: Means are used, irrespective of the distribution, in order to enable comparisons with the Swedish data set

5.6 The associated factors for a low HDLC in the cohort of South African Addison›s patients

The associations of a low HDLC for the entire cohort were youth, low hydrocortisone dose and small dense LDL (Table 30).

Table 30: Comparison of clinical characteristics with respect to HDLC of <1.0 mmol/L

Characteristic	HDLC < 1.0 mmol/L	HDLC ≥ 1.0 mmol/L	*p*-value HDLC <1.0 mmol/L versus HDLC ≥1.0 mmol/L
Age years (IQR)	43.0 (31.5-61.0)	54.0 (40.3-62.0)	**0.04***
Gender Female *N* (%) Male *N* (%)	 47 (57) 35 (42)	 24 (80) 6 (20)	0.06
Ethnicity White *N* (%) Other *N* (%)	 52 (64) 26 (33)	 25 (73) 9 (2)	0.47
BMI kg/m^2 (IQR)	24.7 (21.6-29.8)	24.7 (22.2-30.7)	0.98
Total daily hydrocortisone dose mg (IQR)	20.0 (15.0-30.0)	25.0 (20.0-30.0)	**0.02***

Median: age, BMI and total daily dose of hydrocortisone

BMI: Body mass index

HDLC: High density lipoprotein cholesterol

IQR: Interquartile range

N: Number

$p < 0.05$ considered significant

*$p < 0.05$

In the multivariate model examining the predictors of HDLC <1.0 mmol/L, age and LDL particle size remained independently significant, but hydrocortisone dose was no longer significant (Table 31).

Table 31: Univariate and multivariate Cox hazards regression analyses of predictors of HDLC of <1.0 mmol/L

Variable	Univariate analysis odds ratio (95% CI)	p_1 -value	Multivariate analysis odds ratio (95% CI)	p_2 -value
Age	0.98 (0.96-0.99)	**0.04***	0.97 (0.95-0.99)	**0.04***
LDL species small/ intermediate	4.50 (1.75-1.55)	**0.002***	6.78 (2.17-1.17)	**0.001***
Total daily hydrocortisone dose	0.95 (0.91-0.99)	**0.04***	0.95 (0.93-1.00)	0.05

HDLC: High density lipoprotein cholesterol

LDL: Low-density lipoprotein

CI: Confidence interval

p_1*:* Univariate analysis

p_2*:* Multivariate analysis

$p < 0.05$ considered significant

*$p < 0.05$

5.7 Predictors for elevated triglyceride (TG) levels in the South African Addison's disease patients

The associations for TG >1.7 mmol/L were small/intermediate dense LDL particle size, elevated BMI and increased age (Table 32).

Table 32: Comparison of clinical characteristics, with respect to TG level of >1.7 mmol/L

Characteristic	TG > 1.7 mmol/L	TG ≤ 1.7 mmol/L	*p*-value
Age years (IQR)	38.5 (40.8-70.5)	38.0 (26.8-55.0)	**0.01***
Gender female (%) male (%)	 36 (68) 20 (31)	 44 (68) 26 (41)	0.21
Ethnicity White (%) Other (%)	 39 (62) 23 (37)	 47 (73) 17 (26)	0.20
BMI kg/m^2 (IQR)	27.9 (23.18-31.65)	24.2 (21.0-26.3)	**0.02***
LDL species Small/Intermediate (%) Large (%)	 40 (64) 22 (35)	 15 (23) 49 (76)	**0.0001***
Lipid lowering therapy Yes (%) No(%)	 12 (19) 50 (80)	 6 (9) 58 (90)	0.11
Total daily hydrocortisone dose mg (IQR)	21.25 (15.0-30.0)	22.5 (20.0-30.0)	0.64

Median: Age, BMI and total daily hydrocortisone dose

TG: Triglyceride

BMI: Body mass index

LDL: Low-density lipoprotein

IQR: Interquartile range

N: Number

$p < 0.05$ considered significant

*$p < 0.05$

In the multivariate model examining the predictors of TG >1.7 mmol/L, age and LDL particle size were independently predictive of TG >1.7 mmol/L, but the BMI was no longer independently significant (Table 33).

Table 33: Univariate and multivariate Cox hazards regression analyses of predictors of TG >1.7 mmol/L

Variable	Univariate analysis odds ratio (95% CI)	p_1-value	Multivariate analysis odds ratio (95% CI)	p_2-value
Age	1.02 (1.00-1.04)	**0.02***	1.03 (1.01-1.05)	**0.02***
BMI	1.09 (1.01-1.17)	**0.02***	1.02 (0.95-1.11)	0.58
LDL species Small/ Intermediate Large	5.94 (2.73-12.93) 1	**<0.0001****	4.70 (1.89-11.70) 1	**0.001***

TG: Triglyceride

BMI: Body mass index

LDL: Low-density lipoprotein

p_1: Univariate analysis

p_2: Multivariate analysis

CI: Confidence interval

$p < 0.05$ considered significant

*$p < 0.05$

**$p < 0.0001$

The atherogenic profile of a low HDLC, higher TG and small dense LDL is similarly constituted in Addison's disease patients when compared to healthy subjects.

5.8 Discussion

5.8.1 Summary of findings

The findings of this study may be summarised as follows:

1. The lipid profiles of the South African patients with Addison's disease were highly variable, reflecting a heterogeneous group. Although few (n = 19) of the patients were on lipid-lowering therapy, of these, 88% had a TC of ≥5 mmol/L and 81% had a LDLC of >3.0 mmol/L, indicating incomplete control. In the entire group of Addison's patients, almost 50% had hypertriglyceridaemia, defined as TG >1.7 mmol/L, since less than 1.7 mmol/L is considered ideal. Approximately 65% had hypercholesterolaemia (>5.0 mmol/L) and about 75% had low HDLC (<1.0 mmol/L), with many individuals having strikingly low levels. Approximately 75% had higher than ideal LDLC (>3.0 mmol/L). The proportions in these categories were similar in white and mixed ancestry sub-groups. Other ethnic groups were too small to analyse.
2. Among the diabetics, there was an increase in moderate hypertriglyceridaemia and an increase in severe LDL hypercholesterolaemia. Diabetic patients not receiving lipid-lowering therapy were no different in their manifestation of dyslipidaemia from those that were receiving lipid-lowering therapy, indicating only partial correction. Peculiar to the mixed ancestry subjects, diabetes was associated with higher LDLC concentrations (Table 25).
3. Ethnic differences were identified. White patients with Addison's disease had decreased HDLC, increased small LDL and increased hs-CRP, compared to white controls. In the mixed ancestry patients, increased TG, decreased HDLC, both small LDL and hs-CRP were increased, compared to their mixed ancestry control subjects.
4. GC treatment appeared to influence some of the metabolic parameters. It raised HDLC and glucose, but lowered TSH. Relating dose of GC to

body size, TG and hs-CRP were negatively correlated, but HDLC was positively correlated.

5. When comparing the South African sub-group of Addison's patients with the matched Swedish sub-group, the former group displayed higher TG, TC and LDLC, but lower HDLC. However, importantly, they were managed on lower hydrocortisone doses.
6. A large portion of patients and their respective controls did not achieve recommended lipid and lipoprotein NCEP ATP III targets, as analysed in the sub-group on lipid-lowering therapy and those not using lipid-lowering therapy. A greater proportion of white patients, compared with their controls failed to achieve an HDLC >1.0 mmol/L.

Some of the cardinal findings and other issues are discussed below.

5.8.2 The dyslipidaemias

The wide range of dyslipidaemias identified within this cohort is not surprising, given the variability of environmental and genetic factors, as well as secondary influences, which can modulate the lipid profiles. Evaluation is further complicated by some patients' treatment. The doctors who referred their patients for the study did not assess them for causes and treatment of dyslipidaemia, so that detailed analysis on this topic is not possible. As discussed below, there are also limited data on healthy free-living individuals' lipid profiles in South Africa. Additionally, an effort was made to obtain the best possible controls for patients, but this matching process was imperfect as the controls were selected from the blood donor clinic, which represents a restricted pool from which healthy subjects could be chosen. Taking these difficulties into account, the findings of interest are discussed below.

5.8.3 Normative lipid data in South Africa

A number of community-based studies for each of the white, mixed ancestry, Asian and black groups have been undertaken in South Africa.[18 24 26 27] These

studies were relatively small and were also conducted a long time ago. Since that time, many socio-economic changes have occurred. Although it could be said that there are no current normative values for appropriate and comprehensive comparisons, the CORIS, CRISIC, BRISK and Mamre studies were the only studies available to contrast patients with Addison's disease to people without the disease. Additionally, other controls were enrolled to match with the patients in a more deliberate fashion to ascertain whether real differences exist.

The mean HDLC was considerably lower in the white Addison's disease subjects compared to healthy controls. This could powerfully raise the risk of accelerated atherosclerosis within the usual population range of LDLC. Among white patients, the fundamental difference was that the hs-CRP was higher than their controls. When the mixed ancestry participants in the South African Addison's study were compared to their respective controls, the most important differences were higher TG, hs CRP concentrations and lower HDLC levels in the patient population compared to their controls. These changes promote atherosclerosis.

5.8.3.1 The importance of low HDLC

The finding that 75% of the cohort had an HDLC of <1.0 mmol/L is striking. This finding is generalised across the ethnic spectra. A systematic problem such as aged or improper handling of samples from the Addison's patients may apply, but the same laboratory was also used for controls, making the method used an unlikely explanation. HDLC metabolism is complex and is altered by the acute phase response. Low levels of HDLC have been found in critically ill patients with adrenal dysfunction. ApoAI concentration is also reduced, along with HDLC in the acute phase.[28] Adrenal function relies, at least in part, on HDL as the source of cholesterol for cortisol synthesis.[29] Acute phase response has been shown to increase HDL catabolism, producing a decreased plasma HDL level. A higher TG concentration also occurs typically in the acute phase response, due to decreased lipoprotein lipase activity. This can promote exchange of TG

into HDL and subsequent lipolysis by hepatic lipase, accounting for lower HDLC concentration and small particle size.[28 30] A plausible mechanism for the observed reduction in HDLC was offered by Beentjes et al. He showed that hypopituitary patients who were not supplemented with growth hormone, and were evaluated before and after receiving GC replacement, had a decreased plasma cholesterol esterification, and thus, HDL production could be impaired in Addison's disease patients. The action of GC replacement to decrease LCAT activity largely explains this phenomenon.[31]

Other endocrine deficiencies or factors may also influence lipoprotein metabolism. Hypothyroidism does not explain the low HDLC concentration in this Addison's disease cohort, since the majority (82%) were euthyroid. Only a minority of patients were diabetic, making this disorder an unlikely contributor to low HDLC. Smoking has been found to reduce levels of HDLC, but the prevalence of smoking was only 8% in this cohort, and thus, cannot account for the very low HDLC levels. Nevertheless, smoking cessation should be encouraged as HDLC may be increased by 0.1 mmol/L.[32] Although visceral obesity is a powerful determinant of low HDLC,[33] this parameter was not evaluated specifically. The patients and controls were matched for BMI, which is a guide to visceral obesity, making this parameter an unlikely explanation for the reduced HDLC. It is known that HDLC is lower in the male gender, and for this reason, HDLC was analysed separately in males and females. No gender differences were found in this study. Other influences such as TG, dysglycaemia, alcohol consumption (not assessed in this study) and postmenopausal status should also be considered.[34]

Despite the expected pharmacological consequences of GCs of raising HDLC,[8 10 31 35-41] strikingly low HDLC was observed. Nevertheless, a positive correlation between hydrocortisone dose and HDLC was found in this study, however, HDL still remains responsive to GC doses, indicating that there is an explanation for the low HDLC. It may also suggest that patients were on inadequate doses of

hydrocortisone, but there is insufficient evidence for this. Particularly in elderly subjects, endogenous cortisol levels were also positively correlated with HDLC.[42] In the univariate analysis of the South African Addison study, lower age, lower total daily hydrocortisone doses and increased small dense LDL were associated with low HDLC. The latter is a well-known association. Younger age may be associated with different underlying aetiology of Addison's disease, although it is not obvious why this should influence HDLC (Chapter 4). In the multivariate analysis that incorporated these factors, hydrocortisone dose was no longer independently associated with a decreased HDLC. On the other hand, the small dense LDL is likely to have an association with low HDLC due to the metabolic mechanism(s), rather than being causal, because TG exchange will provide a mechanism for both particles to be modulated by hepatic lipase.[43]

The explanation for the protective mechanism of HDLC against atherosclerosis lies in its reverse cholesterol transport action. In this action, cholesterol is transferred from the peripheral cells to the liver, where cholesterol, as well as its product bile acid, are ultimately excreted into the gastrointestinal tract.[44] Additionally, HDL has anti-inflammatory properties, and thus, acts to reduce atherosclerosis. Experimentally, endothelial function is also modulated by HDL.[45] Visceral obesity appears to be a powerful influence on HDLC. A meta-analysis revealed an increase of 0.007 mmol/L of HDLC for every 1 kg of body mass lost. Diets high in saturated and omega-3 fatty acids, but low in carbohydrates, may contribute to increasing HDLC.[33 46] The benefits of mild to moderate alcohol intake are well-established and include the raising of HDL.[47] Recently, pharmacological therapy has been directed at raising HDLC by CETP inhibition, but this did not lower the CVD event rate. HDLC elevation by this mechanism should not be endorsed to raise HDL until further insights are gained.[48]

The low HDLC in patients with Addison's disease is unexplained and certainly not corrected by hydrocortisone, even though it influenced the HDLC concentration

favourably. If this association is confirmed in the future, detailed studies are required to understand the reason for the low HDLC implications of the disease and CV prognosis.

A low level of HDLC is an independent risk factor for the development of CVD. HDLC levels of <1 mmol/L are strongly predictive of CVD events.[49 50] A reduction of HDLC by 0.03 mmol/L, increases the risk of a CVD event by 23%, and by implication, the low HDLC on its own could accord a 20% increase in CVD events in this South African cohort of Addison's patients.[51] A low HDLC concentration has also been recognised as an independent CVD risk factor by the NCEP, which advises a HDLC threshold of <1.03 mmol/L as a potential target for therapy.[20]

5.8.3.2 The finding of hypertriglyceridaemia among South African Addison's disease patients

Half of the South African Addison's disease patients had TG concentrations that were not ideal, representing another independent risk factor of CVD.[52] There are multiple studies corroborating the direct link between an elevated TG concentration and CVD. In the Prospective Cardiovascular Münster Heart (PROCAM) study, 4 849 middle-aged men were followed for up to 8 years, and a significant association between coronary heart disease and TG, independent of LDLC and HDLC, was found.[53] A meta-analysis of 21 population-based prospective studies, involving 65 863 men and 11 089 women has provided credence to the dogma that a relationship exists between CVD and TG levels. In this relationship, each 1 mmol/L of TG conferred a 32% and 76% increase in coronary heart disease risk among men and women respectively, adjusted for TC, LDLC, HDLC, BMI, blood pressure and diabetes mellitus, which are all potential factors known to influence TG.[54] The multivariate analysis of this study of Addison's disease in South Africa confirmed that age and LDL species were independently predictive for a raised TG level. Plasma TG, along with CETP and hepatic lipase, will result in remodelling of LDL to smaller species.[55] Interestingly, obesity, indicated by

BMI, was no longer independently predictive for TG in the study of Addison's disease in South Africa. A population-based survey in the USA confirmed that 30% of participants, in a total cohort of 8 814 who were all older than 20 years of age, had TG levels of >1.69 mmol/L. In participants older than 50 years of age, the prevalence of hypertriglyceridaemia was 42.8%.[56] In addition to age, TG levels are influenced by increasing BMI, waste-to-hip ratio, dysglycaemia, drugs and hypothyroidism.[57] As the majority of the Addison's patients were matched for age and BMI (in the white and mixed ancestry groups), these factors could not account for this elevation in TG. Moreover, the patients with diabetes showed no differences in their lipid profiles compared to the rest of the cohort. Similarly, it is unlikely that the hypertriglyceridaemia is attributable to primary hypothyroidism, as only 5% of the cohort had a TSH above the upper range of normal (4.94 mIU/L). While the metabolic syndrome, renal disease, T2DM and obesity may all result in hypertriglyceridaemia,[58] specific evidence for renal disease in this cohort was not sought, but it was unlikely to be present to a significant extent.

Although increased VLDL production may account for an elevated TG level resulting from hydrocortisone excess,[4 7-10 59] there is no overt evidence for excessive GC replacement in this cohort, as hydrocortisone dose was not associated with TG levels. The presence of small dense LDL remained independently predictive for an elevated TG, but this is likely to be an association by mechanism, rather than being causal.[43]

Certain genetic defects may account for elevated TG levels, for example, apoE2/E2 status, which occurs in 1:50 of the general population, can result in markedly elevated TG levels and low LDLC associated with small dense LDL species upon metabolic stress.[60] When a metabolic stress occurs, lipoprotein lipase deficiency will manifest with elevated TG, low LDLC and small dense LDL, including an especially high dose of hydrocortisone. A genetic deficiency in hepatic lipase will result in moderate increases in TG levels, elevated HDLC and reduced LDLC

concentrations.[61] The aforementioned genetic defects have not been tested in this cohort, and these uncommon or rare genes are neither expected to have a selection bias in this cohort nor influence the average significantly.[62]

The recommended interventions for borderline high TG (1.69-2.25 mmol/L) are lifestyle changes e.g. weight loss, regular physical activity, smoking cessation, restriction of calories and limitation of carbohydrate intake.[20] When the TG levels are 2.26-5.63 mmol/L, HMG-CoA reductase inhibitors may be indicated to reduce non-HDLC, if the person is at high risk. Fibrates are indicated when the levels of TG are higher, having excluded all the usual secondary causes for hypertriglyceridaemia.[54]

5.8.3.3 Small dense LDL among South African Addison's disease patients

A significantly greater proportion of South African Addison's disease patients had small dense LDL, compared to their controls. This may reveal yet another contributory factor in explaining the excess CV mortality associated with Addison's disease. The ease with which small dense LDL can enter arterial tissue, compared to larger LDL particle sizes, may explain its link with the development of atherosclerosis,[63] along with the greater susceptibility to oxidation and the metabolic milieu, which predisposes to small dense LDL. In the Quebec Cardiovascular Study, the combination of LDL size, apoB and LDL had a significant positive predictive value for CVD events, emphasising the importance of small dense LDL, in association with other lipid parameters, as a CV risk marker. When small dense LDL predominates, it confers about a 3-7-fold increased risk of coronary artery disease.[63] Evidence indicates that LDL particle size is not an independent risk factor for CVD, but rather it is its association with low HDLC, high TG and the presence of hypertension, which in combination confer the greatest risk.[64] Similarly, the multivariate analyses in this study of Addison's disease confirm that an independent predictor for both HDLC < 1.0 mmol/L and TG >1.7 mmol/L is a predominance of small dense LDL, substantiating that it coexists with other risk

factors and that a sub-set of Addison's disease patients is at a particularly high risk of CVD.

In one review, the prevalence of small dense LDL was between 30% and 35% in adult men, between 5% and 10% in men older than 20 years of age, and between 5% and 10% in premenopausal women. The prevalence of small dense LDL in postmenopausal women in the same review was 15-25%.[65] The estimated frequency of the putative gene(s) (Mendellian inheritance) related to small dense LDL was 15% in a USA cohort, but small dense LDL occurred in 25% of 61 nuclear families, indicating that the clustering can occur within at-risk families.[66] The prevalence of small LDL particle size among the South African Addison's disease patients was considerably greater than their respective controls (14% versus 3% among age-, gender-, ethnicity- and BMI-matched white patients, and 17% versus 0% among mixed ancestry patients, compared to their respective controls). Among the South African Addison's disease patients, 15% were found to have small dense LDL particle size, and in the corresponding categories as described by Rizzo et al,[65] the prevalence was 12% among premenopausal women, 16% in postmenopausal women and 16% among men over the age of 20 years. Although the prevalence of small dense LDL in the general South African population is not known, it is expected to be about 15%[66] and may double in people with diabetes. In this study of South African Addison's disease, the proportion of patients with small dense LDL was 18% among diabetics not using lipid lowering therapy, raising the possibility that Addison's disease may confer some protection against the formation of small dense LDL in diabetics, or that autoimmune diabetes may have a lower prevalence of small dense LDL.

5.8.3.4 The effect of diabetes on lipid abnormalities

Despite the relatively small number of patients with diabetes in this cohort, the presence of diabetes contributed to some of the abnormal findings. As expected, there was a greater proportion of patients with moderate levels of

hypertriglyceridaemia (>2.3 ≤ 5.0 mmol/L). Elevated LDLC levels of >5.0 mmol/L is somewhat unexpected until a nephrotic syndrome sets in. Although diabetes became a secondary prevention equivalent in 2003,[67] it was only accepted as such by cardiologists in South Africa in 2006.[68] International and local guidelines for the management of lipids were not uniformly adhered to for diabetics in this South African study, irrespective of whether the patients were funded by the state or by private medical insurance. The observation that small dense LDL was increased in the general Addison's subjects, but not in diabetic Addison's disease subjects is of interest, albeit that it constituted a small group. This could be due to a fundamental derangement in lipoprotein modulation, caused by hypoadrenalism and/or its treatment. As indicated in the description of the whole study population, {(small dense LDL among South African Addison's disease occurred in 22 patients (15%)}. There could be interesting differences in diabetics with Addison's disease, favouring T1DM on an autoimmune basis and modulation of diabetes and complications by supra-physiological hydrocortisone therapy.

5.8.3.5 hs-CRP among South African patients with Addison's disease

CRP is an acute-phase reactant protein that dramatically increases with tissue necrosis, as part of a generalised acute-phase response. Increases detected at low levels with highly sensitive assays can indicate CV risk.[69] As far as is known, no previous study has examined hs-CRP status in Addison's disease, except when the influence of DHEA was evaluated. In the study no change in hs-CRP was found and the mean hs-CRP was 1.4 (0.2-13.1) mg/L.[70] The proportion of patients with elevated hs-CRP was considerably greater in the white and mixed ancestry patients compared to their respective controls. The high levels of hs-CRP found in the South African Addison's disease patients did not correlate with age, any of the lipid fractions, BMI, or Framingham risk.

In the white and mixed ancestry Addison's patients, the hs-CRP was higher than controls. GCs suppress hs-CRP directly or may support the immune response in

such a way that the CRP production is down-regulated when a tissue necrotic event is encountered.[38 71] In contrast to rheumatoid arthritis or SLE, Addison's disease does not manifest with an overt inflammatory state.[72 73] Autoimmune primary hypothyroidism is akin to Addison's disease as there is no overt inflammation and much of the process has burned out by the time the diagnosis has been made. Among euthyroid Hashimoto's thyroiditis sufferers, SAA, fibrinogen and ESR were elevated, but CRP was not. Therefore, it is not expected that the autoimmune process of Addison's disease will raise the hs-CRP significantly. In this cohort, it remains difficult to elucidate whether the low-grade inflammatory state reflected by hs-CRP is a consequence of a systemic problem or the Addison's disease per se, or relates to the treatment with GCs.[72]

There are also known ethnic differences with respect to hs-CRP, as African-American women exhibit higher levels than white women. Nazmi et al showed that increased poverty and socio-economic factors may also account for increased hs-CRP levels.[73] Higher levels were documented among mixed ancestry patients compared to white patients, and in the small Asian group, these levels were strikingly elevated, which may represent either ethnic differences or increased inflammation in this study of Addison's disease. Appropriate cut-offs are unknown, as no population-based study of hs-CRP has been carried out in South Africa.

Anecdotal observations from this study revealed that a few patients died soon after recording strikingly elevated CRP. While the reliability of this finding for predicting death in this cohort remains to be determined, it is potentially a useful modality to detect a high risk immunocompromised individual, which a person with Addison's disease represents. This has been corroborated by an analysis of death registers in Norway, in which 10% of deaths were ascribed to infection and secondary to adrenal gland failure per se.[1] Addison's disease patients may present with occult sepsis, as they often fail to manifest with the usual signs and symptoms. In this group of patients, the utility of CRP may be to detect

severe, but unrecognised illnesses that require treatment, including increases of hydrocortisone supplementation. The significance of finding an elevated hs-CRP will need to be investigated in long-term studies of Addison's patients to determine if it translates into accelerated CVD morbidity and mortality.

5.8.3.6 NEFA among South African Addison's disease patients

NEFA was significantly lower in white patients compared to their respective white controls. The association with NEFA release and sympathetic tone is well-established.[74] In many cases of long-standing Addison's disease, adrenal-medullary destruction occurs, making the lower level of NEFA that was found in our cohort, compared to their controls, not altogether surprising.[75] Though hormone-sensitive lipase may not be suppressed in diabetics, leading to increased NEFA flux to the liver and consequent overproduction of VLDL, diabetic white and mixed ancestry subjects did not have higher NEFA concentrations compared to non-diabetic Addison's patients (Table 26). There could be blunting of NEFA release in Addison's disease. Despite the lower levels of NEFA in Addison's patients compared to controls, NEFA was positively associated with TC and LDLC. Plasma cholesterol esterase activity is also positively correlated with TC and LDLC. Therefore, it is entirely plausible that ongoing enzymatic action of cholesterol esterase could have continued in the samples of the Addison's patients that may have been incorrectly frozen to account for the NEFA association with TC and LDLC.[23] Low concentrations and mismatch of GCs to stress could influence the endothelial cells to promote atherosclerosis in the setting of co-existing risk and contributing factors. While NEFA levels are correlated with CVD death, the findings in this study demonstrate levels that are lower than healthy control subjects. However, it has not yet been determined whether a low NEFA concentration is protective against CVD. Even if this is proven to be the case, the balance of CVD risk factors may still count against Addison's disease subjects.

5.8.3.7 Comparison of the lipid and lipoprotein data of Swedish and South African Addison's disease patients

South African Addison's disease patients demonstrated an adverse lipid profile. They also took lower replacement doses of hydrocortisone compared to their Swedish counterparts. Relative to the Swedes, the South African cohort had higher TC (13%), TG (32%), LDLC (39%) and lower HDLC (137%) levels (Table 30). Whilst some of these changes may be within the range of variation of laboratory analyses, the TG, LDLC and HDLC differences are striking. The only expected difference was in TG concentration, because unlike the Swedes who fasted, the South African cohort had random samples. This is the first study to examine the lipid profiles of patients with Addison's disease in two geographically distinct cohorts. The adverse profile documented in the South African Addison's disease patients is seen despite careful matching of patients with respect to age, gender, ethnicity and BMI. The prevalences of smoking, hypertension, diabetes and lipid-lowering therapy were similar in both groups, but it is uncertain to what extent lifestyle, socio-economic status, genetics and levels of health-care factors may have contributed to this adverse lipid profile.

A potential weakness of this sub-study is the fact that the lipid analyses were conducted in two separate laboratories. Although the differences in the results between the two laboratories were not analysed by exchanging samples, results should be comparable by using internationally accepted calibrators. The differences between the different laboratories in Sweden and South Africa should be small if proper quality control was exercised. The University of Cape Town laboratory used Precinorm (Boehringer Mannheim GmbH, Mannheim, Germany), which has international consensus values for plasma lipids, but the magnitude of the potential difference is acceptable at 10% for the range of accepted readings, as stated in the package insert. Compared to the other lipid fractions, the potential variability in HDLC is the greatest and it is also likely to contribute to a greater difference, than the other lipid fractions. This variability,

as well as that in non-fasted TG, may also influence the LDLC calculation. The heparin-Mn chloride methodology of HDLC evaluation produces lower values, compared to homogenous assays used in automated machines. The magnitude of variance between the two cohorts is too great to be explained by the differences in methodology alone. A prospective study using a single laboratory is advised in the future, to confirm and investigate this finding.

The prevalence of primary hypercholesterolaemia and significant hypertriglyceridaemia is 14% and 3% respectively in the sub-group of South African patients that were matched for the Swedish patients. Interestingly, the mean TC identified in the Swedish cohort is considerably more favourable compared to the MONICA population-based survey in Northern Sweden.[76] Nevertheless, it appears that the South African Addison's disease patients have a greater proportion with neglected levels of cholesterol. Poor lifestyle may significantly contribute to the dyslipidaemia and CV risk. Reddy et al predicted a rise in burden of CVD in the Third World, in association with early-onset vascular events.[77] The Scandinavian countries by comparison, have enjoyed long-standing emphasis on reducing CV risk factors. The 4S study has been pivotal in raising awareness and detection of lipid abnormalities in Northern Europe,[78] and could have influenced the Swedish population favourably. Mass screening of patients in South Africa for lipid abnormalities has not been encouraged and it represents a deficiency in this health system. Specifically, lifestyle factors such as exercise and diet were not analysed in this study, but it is conceivable that in First World countries, greater willingness exists among the public to pursue physical activity and healthier food options.

It is possible that the higher doses of GCs in Sweden, relative to the South African cohort, may increase the HDLC through a reduction of hepatic lipase and CETP activity.[39] South Africa has been identified as an important geographical area for genetic dyslipidaemias and the known prevalence is in the order of 1% in white

Afrikaans-speaking citizens.[79] Thus, by random selection and by virtue of the sporadic nature of Addison's disease, finding a significant number of patients with genetic dyslipidaemias among this small cohort is unlikely. The observation that the South African cohort had, on average, a lower exposure to GCs, could not account for the wide array of lipid abnormalities. In the Swedish-South African comparative study, the proportion of subjects treated with lipid-modifying drugs was similar in the two groups and could not clarify the differences seen in these respective lipid profiles. It is possible that the South African and Swedish cohorts may include minor genetic traits, which could potentially influence the lipid profiles. However, this again is not subject to selection bias. The difference found between the Swedish and the South African Addison's disease patients is most likely explained by the economic and lifestyle differences. The diet is likely to be different between the Swedish and the South African cohorts, with the South African cohort consuming more saturated fat and cholesterol.[80-82] Lack of education, economic reasons and availability of certain foods may account for this difference. Detailed dietary questionnaires in the future may assist in unravelling the reasons for these striking differences.

5.8.3.8 Cardiovascular risk

The way that an artery responds to an atherogenic process that involves lipoproteins and oxidative stress could be influenced by Addison's disease per se. Cholesterol esterase may have modified NEFA concentration in vitro, but its effect in vivo should also be considered, as it influences the processing of lipoproteins and sphingomyelin-ceramide signalling systems. Not only is cholesterol esterase expressed in endothelial cells,[83] but it may also have a GC response element.[84] Therefore, as atherosclerosis is an inflammatory process, a deficiency of GCs, as occurs in undertreated Addison's disease, may also be influential in promoting atherosclerosis.

The findings of this study may explain the findings of an earlier single study emanating from Sweden. In this study, Addison's patients on stable doses of hydrocortisone replacement have twice the CVD mortality compared to the background population.[2] The lipid and lipoprotein data of patients disclose variable, but often atherogenic profiles, which compared unfavourably with their respective controls and limited South African normative data. Additionally, compared with Swedish Addison's disease patients that were matched for gender, ethnicity and BMI, South African Addison's disease patients had considerably more atherogenic lipid profiles. The Framingham risk was negatively correlated with hydrocortisone doses, which suggests that hydrocortisone may either ameliorate risk or that a lack of hydrocortisone promotes risk.

This is one of the first studies that attempts to determine the reason(s) for the significant CVD mortality among Addison's disease patients. In a recent study,[85] Addison's patients, compared to controls whose glucose levels were no different, had a higher proportion who were hypercholesterolaemic (18% vs. 8%) and had hypertriglyceridaemia (18% vs. 8%). However, none of these Addison's patients demonstrated an HDL of <1.036 mmol/L or an LDL of >5.51 mmol/L, which is in sharp contrast to this South African Addison's disease study, in which a significant proportion had either abnormally elevated LDL or reduced HDL levels. Gurnell et al[86] investigated the effect of DHEA supplementation in patients with Addison's disease and did not find abnormalities in lipid profiles before or after DHEA supplementation. The combination of small dense LDL, low HDLC and an elevated TG has been designated as the atherogenic lipoprotein phenotype.[85] Both white and mixed ancestry patients had more small dense LDL compared to local controls. Whilst there was no significant difference in TG in white patients compared to controls, the mixed ancestry patients displayed raised TG. Both white and mixed ancestry patients, when compared to controls, displayed low HDLC. It is thus possible that the atherogenic lipoprotein phenotype applies to atherosclerosis risk in Addison's disease.

The proportion of the cohort with Framingham risk of >20% CVD risk in 10 years was 36%. The average cohort 10-year interval risk of CVD for a median age of 46 years was 13.3% and was worse for diabetics (25.4%). This is considerably greater than the available comparative data from the USA, which reflects a 8% risk for men and 3% for women in the age group approximately 46 years of age.[13] The Framingham risk is derived from data that was probably collected in the absence of Addison's disease and it may not be applicable to Addison's disease patients. Judging from the increased mortality in the Swedish study,[2] the risk calculation is likely to underestimate CVD. Although the Framingham risk prediction has not been formally validated in South Africa, the prediction will reflect the trend, even if it lacks accuracy. In a follow-up study of the Framingham cohort, hs-CRP predicted the risk of death, but not of major CV events, and only showed moderate improvement over the conventional risk factors.[87] A few patients in this South African Addison's disease study with severe elevations of CRP suffered imminent death, the causes of which were likely to be conditions other than vascular disease, although in one patient, the cause was due to a cerebrovascular accident. It is therefore questionable whether hs-CRP in Addison's disease is reliable for predicting CV risk.

It remains difficult to unravel the precise cause or causes for the atherogenic dyslipidaemia in this cohort. There are multiple factors that could account for increased CVD. It is clear that Addison's patients demonstrate multiple CV risk factors, but only CV endpoint and mechanistic studies can confirm this. Addison's disease patients with moderate dyslipidaemia should be considered for lipid-modifying therapy, as they may have a higher than expected CVD risk than predicted by the Framingham risk scores. Other parameters such as vascular imaging should be used as an adjunct to assessing risk. While the HDLC was lower than expected, there is a correlation with total daily hydrocortisone dose and this may need mechanistic and therapeutic assessment. The changes in lipids and lipoproteins may partly be attributable to either an excess or a deficiency of

hydrocortisone, but several other influences could also be operating.

There is sufficient evidence, based on this Addison's disease study, for clinicians involved in the management of patients with Addison's disease to consider that they are at a higher than average risk of CVD. Although the Framingham risk for CVD may be determined by meticulous assessment, this may be an underestimate. Pharmacotherapy permits the treatment of risk factors, for example, hypertension and hypercholesterolaemia, which should be used in conjunction with lifestyle modification.[88] In addition, evaluation of the lipid and lipoprotein profiles should be a fundamental component of care for patients with Addison's disease. This particularly applies in the South African context, where dietary indiscretions may contribute to some degree to the adverse lipid profiles; healthy eating habits should be taught to this sub-set of patients. The longitudinal effect of reducing lipid abnormalities by using lipid-modifying therapy, has not been undertaken in Addison's disease, but there is sufficient circumstantial evidence to warrant its use, when it is indicated. The sub-group of Addison's patients using lipid-lowering therapy failed to achieve lipid and lipoprotein targets, and so remain at risk for a CV event. This is alarming, as the CV risk may be underestimated using conventional CV risk factors, and so by implication, Addison's disease patients as a group would necessitate intensive modification of CVD risk factors. Treatment of other risk factors such as hypertension, diabetes and encouraging smoking cessation should be the routine.

It is concerning to note that the hs-CRP was higher than control subjects, and this on its own should necessitate vigilant attention to modifiable CV risk factors, even though it may not be applicable due to altered immune status. The anecdotal observation that a minority of patients died soon after an elevated hs-CRP was assayed, may compel it to be routinely assayed at the time of inter-current illness or concern about health in general, but currently its recommendation would be based on conjecture, rather than powerful scientific evidence.

5.8.3.9 Future directions for the management of dyslipidaemia and cardiovascular (CV) risk of South African Patients with Addison's disease

Addison's disease is rare and primarily managed by endocrinologists, who should be conversant with dyslipidaemia, but may not be trained for atherosclerotic risk inferences. The initial consultation is crucial in order to establish baseline clinical and biochemical parameters. Given the relatively slow evolution of atherosclerosis, time is available to re-evaluate patients following correction of hormone deficiencies. It is sound clinical practice to introduce lipid-lowering therapy early among patients with Addison's disease, considering that the life expectancy is generally good. This contention is supported by the work in this area that demonstrates that Addison's disease patients have increased (twice) the CV risk compared to healthy individuals. The applicability of the Framingham risk calculations for CVD may not be perfect and may underestimate the true CVD risk because other factors, aside from those used in the Framingham calculations, may operate. With detailed information about CV outcomes in large cohorts, a correction factor could be derived to enhance the accuracy of the Framingham or other risks scores.

A prospective observational study to examine the effect of addressing CVD among Addison's disease patients would require an extremely large sample size, because Addison's disease is uncommon and atherosclerosis is multi-factorial in pathogenesis. Surrogate markers, for example, intimal-medial thickness evaluation, are useful adjuncts to standard CV disease risk factors, but again may not be similar in their implications owing to changes that may occur, due to Addison's disease per se or its cause. The longitudinal population-based studies examining carotid intimal-medial thickness (CIMT) of >1 mm and/or discrete plaques to subsequent myocardial infarction event rates from Finland, Italy, USA and Holland, indicate that the relative risk varies from 2.1-6.3, and the greatest risk is exhibited in individuals with focal plaque.[89] This is likely to apply in Addison's disease, but this technology uncommon in South Africa, which limits the utility for

assessment of Addison's patients for CVD risk. Thus, it becomes more important to harbour awareness and attention to modifying conventional CV risk factors in South Africa.

The retrospective data available for study in this thesis indicate that CV risk and dyslipidaemia may be increased in a similar way to that found in Sweden. Although this study did not show a relationship between hydrocortisone dose and lipid abnormalities, there are no specific recommendations with respect to hydrocortisone supplementation and dyslipidaemias. Management of an acute CVD event in Addison's disease is also not directed by previous studies. The lack of an acute cortisol response to such a stress may also influence outcome. It is not known whether Addison's disease patients are at greater risk of acute mortality in the context of an acute coronary event, and therefore this represents an area of research that should be undertaken in the future.

It still remains uncertain to what extent the atherosclerotic lesion of the vessel wall is modulated, by either insufficient or excessive hydrocortisone doses. While one author suggests that there is no change in endothelial flow-mediated dilatation in response to GCs,[91] the question of whether Addison's patients have abnormal physiological responses to flow represents an area of possible further research. It is clear that Addison's patients will require support of at least tertiary care to ensure the most favourable outcome for CVD. The role of cholesterol esterase should be explored for its activity in Addison's disease, its response to GC replacement and possible atherogenesis.

5.8.3.10 Conclusions

This is the first comprehensive study in South Africa that examines the lipogram and markers of CV risk in Addison's disease patients, in order to verify the excessive CVD risk shown by mortality among patients in Sweden with Addison's disease. A number of lipid abnormalities, as well as markers of CV risk, have

been demonstrated. HDLC is markedly abnormal in this cohort of patients, but this requires confirmation and further research for its implications. Whilst some patients were treated (albeit not to target) for LDL, more can be done to achieve target values in the current guidelines. Pharmacological therapy directed at modulating HDL is still under investigation. A local registry is recommended for South Africa to increase its awareness of dyslipidaemia and risk.

5.9. References

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Chapter 6
Monitoring hydrocortisone replacement therapy using salivary cortisol day curve measurements

6.1 Introduction

Salivary cortisol is a non-invasive, accurate measure of endogenous free cortisol production. It has been utilised in fields such as sports physiology, endocrinology, immunology and psychology,[1] and has demonstrated good correlation with serum in Addison's disease.[2 3] Therefore, it would be interesting to determine the degree of salivary cortisol exposure in Addison's patients on their usual hydrocortisone replacement versus healthy controls, and to correlate this level with the evolution of side-effects.

Moreover, it has not yet been ascertained which time point after hydrocortisone ingestion is the most reliable in predicting salivary cortisol peaks. Following hydrocortisone ingestion, supra-physiological peaks of serum and salivary cortisol are generated in patients with Addison's disease and hypopituitarism, in contrast to healthy controls' endogenous levels.[4-10] It is uncertain whether the total exposure to cortisol (AUC) or the supra-physiological peaks are responsible for the deleterious effects of hydrocortisone.

6.2 Aims

The purpose of this study is to test the hypothesis that patients with Addison's disease, who are fully replaced on hydrocortisone and fludrocortisone, may be subject to supra-physiological doses of GCs, as examined using salivary cortisol AUC.

The specific objectives are:

i. to determine the salivary cortisol AUC for patients with Addison's disease on full replacement and compare these results to healthy control subjects' endogenous salivary cortisol concentrations
ii. to determine the optimum time to sample the salivary cortisol in patients and healthy controls that would reflect most accurately the salivary cortisol peak and the AUC
iii. to determine whether an increased salivary cortisol AUC translates into abnormal lipids, lipoproteins and markers of CV inflammation

6.3 Patients and methods

The methods are described below under the headings of patients, procedures, salivary cortisol test principle, expected cortisol levels in saliva, automated measurement of salivary cortisol, collection method for salivary cortisol and statistics.

6.3.1 Patients

The patients enrolled in the Addison's disease cohort, described in Chapter 3, were eligible for inclusion in this study.[11] Approval from the University of Cape Town Research and Ethics Committee was obtained for this sub-study. All participants in the South African Addison's study were sent a letter that explained the purpose of this study and 31 patients with Addison's disease agreed to participate. Healthy control subjects, comprising mostly medical students, were recruited through the university website. Both participants and healthy control subjects were excluded if they were pregnant, using medication that could interfere with salivary cortisol analysis or taking hormonal therapy. Examples of medications that were deemed to be an exclusion criterion included oestrogens or hormonal therapy, histamine-2 receptors antagonists, antidepressants and asthma medication.[12] All participants signed written informed consent forms.

6.3.2 Procedures

Since the salivary cortisol study was performed in both the patients' and the healthy control subjects' homes, counselling was performed both telephonically and by letter to ensure the maximum adherence to the study protocol. The saliva was obtained using a straw and drained into a 1.5 ml microcentrifuge tube, by passive drool technique.

The patients and controls were asked to collect their saliva at 08h00 (immediately before the first hydrocortisone dose in the case of patients), at 08h30, 09h00, 09h30, 10h00, 10h30, 11h00, 12h00, 14h00, 16h00, 16h30, 17h30, 19h00, 21h00, 22h00 and at midnight (16 samples per participant) in a home environment. Patients were asked to continue their usual hydrocortisone maintenance therapy, but to indicate the precise times of these doses. In order to prevent contamination with food and drink, participants were advised only to eat at certain times of the day, that is, have breakfast at 06h45 for 15-20 minutes, lunch between 12h30 and 13h00, dinner between 19h30 and 20h00, and not to snack at other times or drink alcohol for 24 hours before collecting saliva and on the day of collection. In addition, the participants were requested to avoid dairy products, for example, cheese, yoghurt and milk. Fizzy drinks, oranges, lemons, pineapples, sweets and chocolates were also to be avoided. Participants also had to limit brushing their teeth, as there was a chance that injury to the gums could allow blood to pass into the saliva collection and falsely increase cortisol levels. Similarly, chewing gum or bubblegum or flossing teeth was not permitted during the day of sampling. Participants were presented with sealed straws for maintaining hygiene and were advised to cut the straw 6 cm from the pointed edge. They were then asked to chew on the back of the straws to encourage saliva production and pass as much saliva as possible through the straws while their head was facing forward and down to the floor. Each microcentrifuge tube was marked with the patient's initials and the time point of each collection. The participant was advised to use a new straw and tube for each collection, recording the time point for each one, to

prevent contamination with a previous sample.

In order to enhance adherence to the protocol, patients and control subjects were told that they would be contributing significantly to science and medicine. A review in 2000 by Shumaker et al suggested ways to promote adherence in clinical trials through the combination of a written record and informing each of the subjects of the importance of their contribution.[13]

All participants were counselled regarding measures to prevent contamination with blood, for example, brushing teeth at designated times, followed by oral rinses. On completion of the collection, the participants were advised to refrigerate or freeze the samples. The samples were returned either by hand or by express mail to limit exposure to room air. The specimens were stored at -70 °C.

6.3.3 Salivary cortisol test principle

The assay used was a competitive electrochemi-luminescence immunoassay that used a sheep polyclonal antibody. Endogenous cortisol in the sample is freed from binding proteins by danazol, which competes with a cortisol derivative (cortisol-peptide-tris bipyridyl ruthenium complex) for the binding sites of biotinylated antibody. The second incubation follows, with the addition of streptavidin-coated paramagnetic microparticles and the entire complex binds to the solid phase. This entire reaction mixture is then drawn into a measuring unit, where the microparticles are magnetically captured onto the surface of the electrode. These unbound complexes are then removed. A potential difference to the electrode is applied, which induces a chemi-luminescent emission and which is quantified by a photomultiplier. The amount of cortisol is determined using a calibration curve. The sample volume for the assay is 20 µl.[14]

6.3.3.1 Expected cortisol levels in saliva

The following normal reference values contained in the package insert were

provided with the Elecsys (Roche) cortisol assay kit, having been derived from 154 healthy individuals and sampled in the morning hours between 08h00 and 10h00, and 14h30 and 15h30, revealed ranges of 1.90–19.1 nmol/L and 2.05–11.9 nmol/L respectively. A population-based study to determine the reference range in the normal population in South Africa was not undertaken as part of this investigation.

6.3.3.2 Automated measurement of salivary cortisol

The Elecsys (Roche) random access analyser has been shown to offer good performance in the low nmol/L range that is required for evaluating salivary cortisol and it demonstrates low cross-reactivity with cortisone (0.3% at 2.7 µmol/L). The lower limit of detection for salivary cortisol is 0.5 nmol/L and a 20% inter-assay c.v. was reported, using this methodology.[14]

6.3.4 Collection method for salivary cortisol

In this study, the salivary cortisol samples were collected by the passive drool technique, because the literature suggests that it has a superior yield for salivary cortisol compared to salivettes.[15] However, salivettes were recommended as the preferred collection device in the assay package insert. For this reason, the correlation between salivary cortisol collected in microcentrifuge tubes by the passive drool technique, by salivettes, and by passive drool technique and instilled on to salivettes, was examined. The degree of agreement was determined in ten healthy control subjects, providing three salivary samples collected sequentially at 08h00, using the aforementioned collection methods. There was a poor correlation between salivary cortisol obtained from microcentrifuge tubes directly, compared to salivary cortisol obtained using salivettes (r = 0.37) {personal communication, John Stanfliet, National Health Laboratory Services (NHLS) 2008}. On the other hand, the correlation between salivary cortisol collected in microcentrifuge tubes and instilled onto salivettes was r = 0.96, which although slightly sub-optimal, was considered adequate for comparisons.[16] A bias of 0.68

nmol/L was calculated between these latter two methods for collecting saliva, which is clinically insignificant.

6.3.5 Statistics

Data were analysed using Stata version 11.0 (College Station, Texas, USA.) Salivary cortisol fluctuations are presented graphically for each participant, with grouped comparisons of median salivary cortisol levels at different time points for patients versus controls. AUC and clearance were calculated using WinNonlin® version 6 software, Pharsight corporation Mountainview California USA. The distributions are described as means with standard deviations, medians with interquartile ranges, or percentages, as appropriate. Distributions of continuous variables were compared using student's t-test, replaced by rank-sum tests for non-normally distributed variables. Proportions were compared with Chi-squared tests, replaced by Fisher's exact tests for small samples. Repeated measures ANOVA were used to examine the differences between salivary cortisol concentrations between patients and controls. Spearman's correlation was used to examine the association between the overall AUC for salivary cortisol and cortisol concentrations at different time points. Throughout, 2-sided statistical tests with $p < 0.05$ indicating significance, were used.

6.4 Results

As seen in Figure 20, there were 31 patients who agreed to participate in this study and 113 patients who were not enrolled. Of these, 12 patients were excluded because of concomitant medication, three patients declined to participate in the salivary cortisol study and 98 patients did not respond. The large number of non-responders was attributed to a lack of interest in the study and a largely mobile society, which meant that the postal addresses at the time may have been incorrect. It was suggested that this study may have been excessively rigorous, dissuading potential participants from enrolling. There were 30 healthy control

subjects, who were not matched for any of the patient parameters.

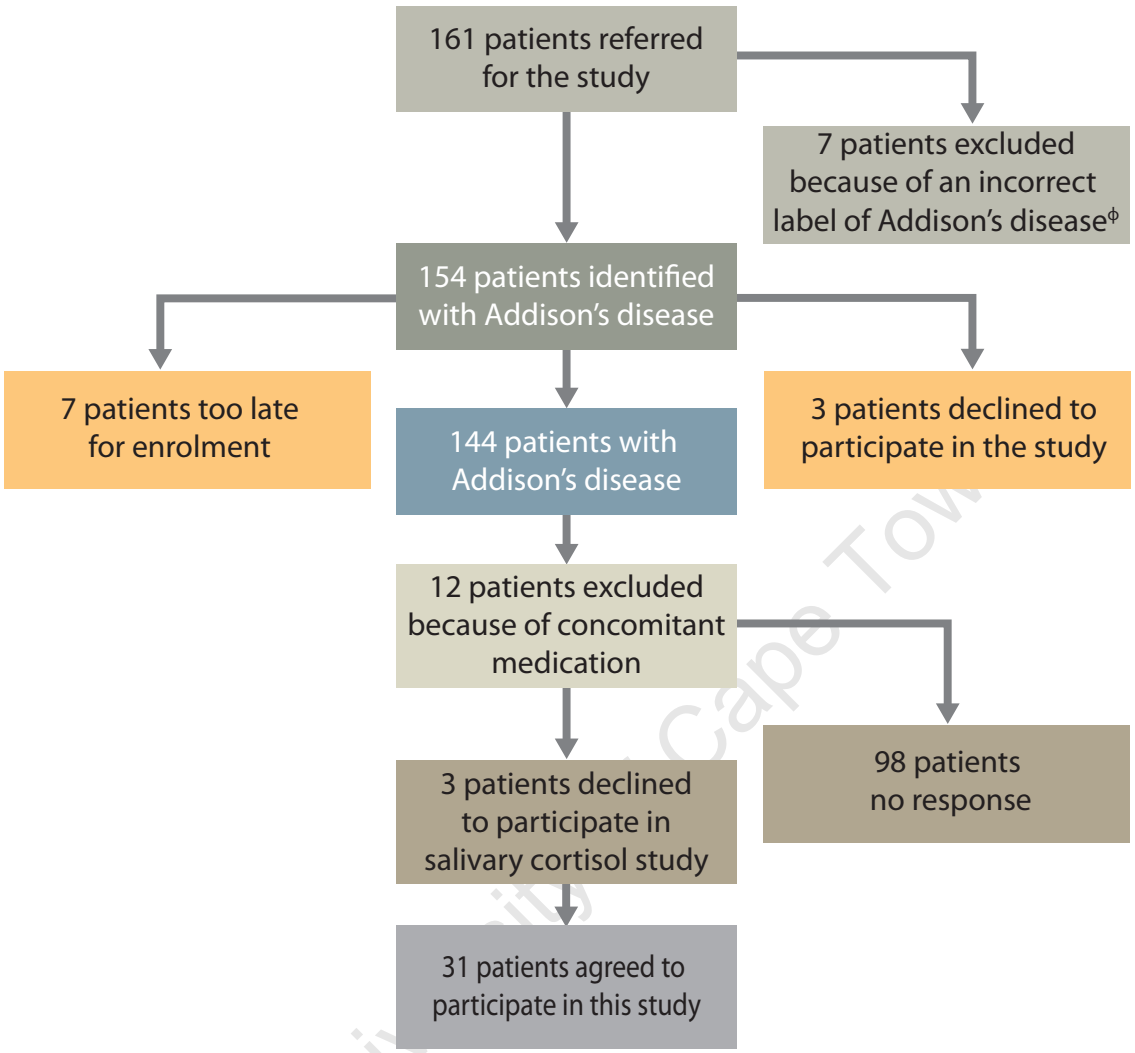

Figure 20: Process for enrolling Addison's disease patients in the salivary cortisol study. Patients were excluded because of concomitant therapy, including sex steroid therapy, depression and using antidepressants, supplemental steroid use for asthma, and anticonvulsants. The reasons for the poor response have not been ascertained, but it is speculated that the lack of interest in the study and a largely mobile society meant that the postal addresses at the time may have been incorrect. It was suggested, that this study may have been excessively rigorous, dissuading potential participants from enrolling.

6.4.1 Baseline characteristics of the Addison's disease subjects who participated in the salivary cortisol sub-study

The participants' baseline data are presented in Table 35. The 30 healthy control

subjects differed from the 31 Addison's patients, as they were younger and there was a predominance of both males and black subjects. The 31 participants of this sub-study were compared to the remaining 113 Addison's patients who did not have their salivary cortisol monitored (Table 34). An equivalent proportion of the sub-study participants was female (74% versus 57%; $p = 0.07$) and the ethnic distribution was different, which can be explained by the absence of black patients participating in the sub-study ($p = 0.0003$). On the other hand, age, total daily hydrocortisone dose, the hydrocortisone dose/m^2 and BMI were no different between these groups.

Table 34: Comparisons between patients who underwent salivary cortisol examination and the remaining cohort of Addison's disease patients

Variable	Salivary cortisol patients *N* = 31	Healthy control subjects *N* = 30	Remaining cohort *N* = 113	*p*-value salivary cortisol patients versus healthy control subjects	*p*-value salivary cortisol patients versus remaining cohort	Total cohort *N* = 144
Age in years (IQR)	48.0 (36.0-62.0)	24.0 (23.0-25.0)	46.0 (32.0-61.0)	**<0.0001****	0.5	46.5 (32.8-61.0)
Gender *N* (%) Females (%) Males (%)	 23 (74) 8 (26)	 13 (43) 17 (57)	 64 (57) 49 (43)	**0.03***	0.07	 88 (61) 56 (39)
Ethnicity *N* (%) White (%) Mixed ancestry (%) Asian (%) Black (%)	 24 (81) 6 (18) 1 (1) 0 (0)	 4 (13) 8 (27) 0 (0) 18 (60)	 69 (61) 29 (26) 4 (3) 11 (10)	**<0.0001****	**0.0003***	 94 (65) 34 (24) 5 (3) 11 (8)
BMI kg/m^2 (IQR)	25.8 (22.5-27.7)	Not assessed	25.0 (22.1-30.4)	N/A	0.23	25.8 (24.8-26.8)
Total daily hydrocortisone dose mg (IQR)	20.0 (20.0-30.0)	N/A	25.0 (20.0-30.0)	N/A	0.14	25.0 (20.0-30.0)
Total daily hydrocortisone dose /m^2 mg (IQR)	12.0 (10.4-14.2)	N/A	14.3 (10.6-17.0)	N/A	0.85	12.8 (10.4-6.8)

Median: Age, total daily hydrocortisone dose, total daily hydrocortisone dose/m^2 and BMI

IQR interquartile range,

N number,

SD standard deviation

BMI: Body mass index

p-value: Comparison of patients who underwent salivary cortisol examination with the remaining cohort of Addison's disease and healthy control subjects.

N/A: Not applicable

$p < 0.05$ considered significant

$^{*}p < 0.05$

$^{**}p < 0.0001$

The individual-timed salivary cortisol concentration plots for healthy volunteers and patients with Addison's disease are shown in Figure 21. It is evident from these that no salivary cortisol measurements were performed between the hours of 00h00 and 08h00, limiting the appraisal of the day curves to 16 hours. Moreover, significant variability is identified for both patients and controls, with the former exhibiting far greater excursions compared to the healthy control subjects' endogenous cortisol concentrations. In the salivary cortisol day curves for healthy volunteers, no consistent peaks were identified at the time points 08h00 and 16h00 when endogenous serum cortisol levels are expected to demonstrate an initial peak, followed by a secondary peak respectively.[17] The CAR was not assessed in this study, as salivary cortisol at the time points 15 minutes, 30 minutes and 45 minutes post awakening were not sampled, which traditionally has been used to investigate psychological influences on the HPA axis.[19]

The median salivary cortisol concentrations during the day curve measurements in patients and healthy control subjects are depicted graphically, without the IQR, as there is considerable overlap at all time points (Figure 22). For the entire day curve, the median salivary cortisol concentration was greater in patients than in controls, but the difference was greatest between these two groups during the interval between 08h00 and 14h00.

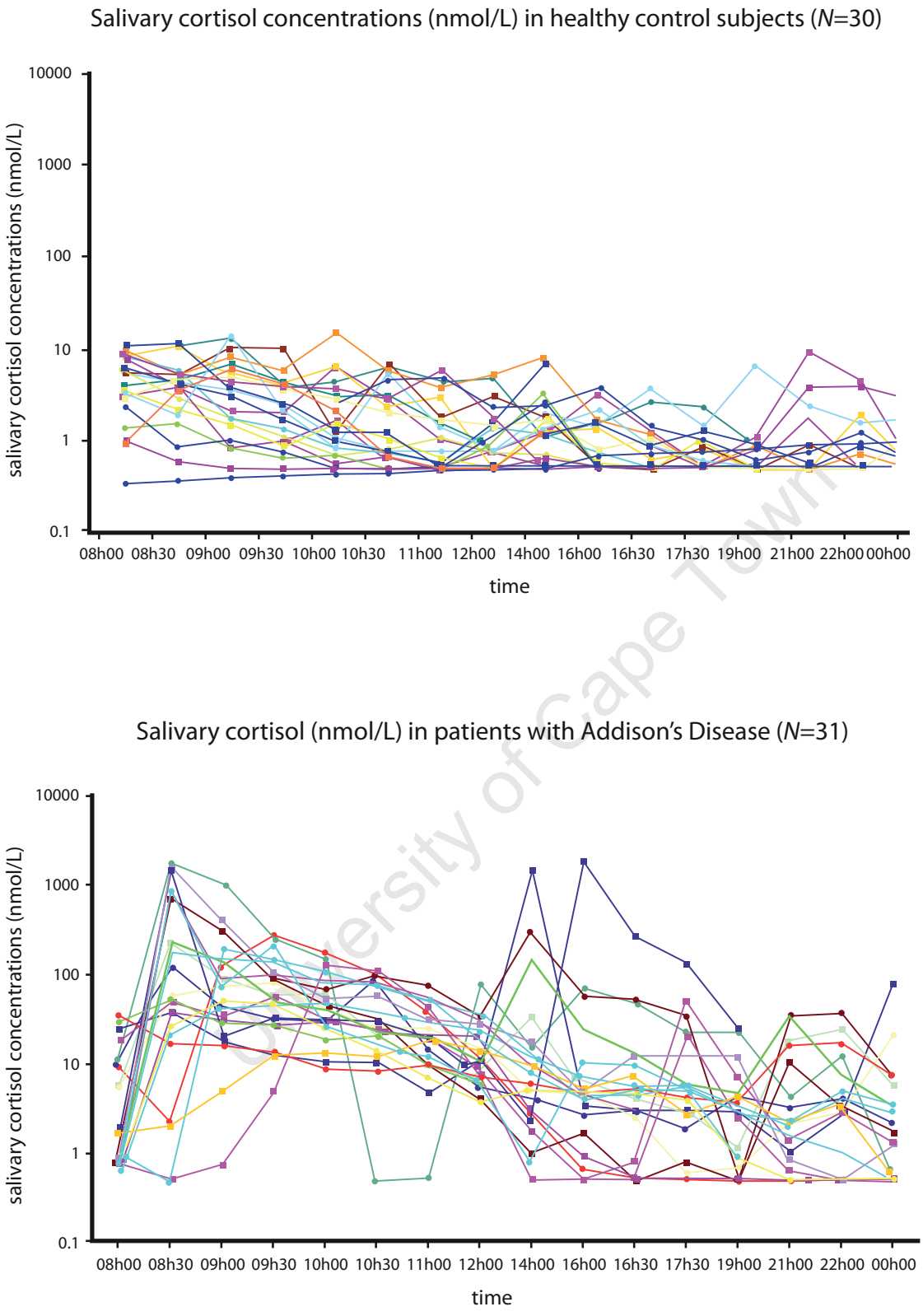

Figure 21: Salivary cortisol concentrations in healthy control subjects and Addison's disease patients

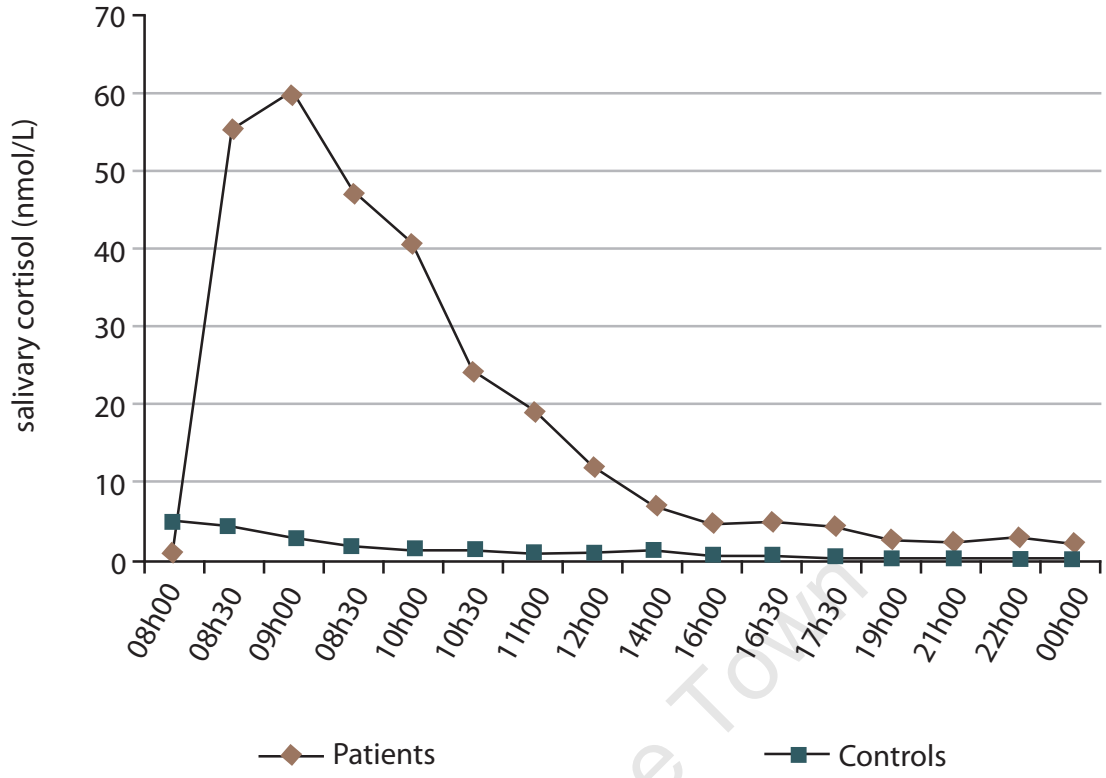

Figure 22: Comparison of the median salivary cortisol in patients and controls. The interquartile ranges (IQR) for each of the time points indicated in clock time are not shown, because of the substantial overlap that exists between patients and controls.

The Addison's patients demonstrated significantly higher first and second peak salivary cortisol concentrations, as well as longer time to peak levels, compared to healthy subjects' endogenous cortisol profiles (Table 35). Considerable inter-individual variability of salivary cortisol concentrations was noted in the spaghetti plots, in both healthy control volunteers and Addison's patients. The median (IQR) salivary cortisol AUC was 15-fold higher in patients compared to the controls. The AUC correlated with the peak cortisol concentrations both in patients (r = 0.87; p = 0.0001) and controls (r = 0.74; p = 0.0001), which is expected as there is ordinarily a strong correlation between AUC and peak concentrations, as shown in the evaluation of different drugs in plasma.[20–22] Moreover, the peak correlates well with the AUC for salivary ofloxacin. Taken together, there is evidence for the use of either peak plasma or saliva levels to indicate an AUC.[23] As expected, the

median (IQR) clearance rates of cortisol were significantly lower among patients with Addison's disease who enrolled in the salivary cortisol study, as 0.02 (0.01–0.05) litres/hour ($L*h^{-1}$) versus 0.37 (0.28–0.45) ($L*h^{-1}$) for the healthy control subjects ($p = 0.0001$).

Table 35: Salivary cortisol excursions in Addison's patients on hydrocortisone and endogenous concentrations in healthy controls

Variable	Patients (*N* = 31)	Control Subjects (*N* = 30)	*p*-value patients versus controls
Peak cortisol (C_{max}) nmol/L (IQR)	174.5 (59.3-837)	6.50 (4.71-9.3)	**0.0001***
Time to peak cortisol (t_{max}) minutes (IQR)	60.0 (30.0-105.0)	30.0 (0.1-112.5)	0.16
First peak cortisol nmol/L (IQR)	174.5 (53.0-754.7)	6.27 (3.90-8.47)	**0.0001***
Time to first peak cortisol minutes (IQR)	30.0 (30-75)	0.1 (0.1-30)	**0.0001***
Second peak cortisol nmol/L (IQR)	18.9 (5.2 to-76.9)	3.12 (1.76-4.79)	**0.0001***
Time to second peak minutes (IQR)	510.0 (480.0-840.0)	480.0 (360.0-615.0)	0.05
Trough cortisol nmol/L (IQR)	<0.50	<0.50	
Time to first trough minutes (IQR)	480.0 (0.1-660)	405.0 (180-570)	0.56
AUC nmol*min*L^{-1}	390.1 (177.1-928.9)	21.4 (14.6-28.4)	**0.0001***

Median: Peak cortisol, time to peak cortisol, first peak cortisol, time to first peak cortisol, second peak cortisol, time to second peak cortisol, trough cortisol, time to first trough and AUC

AUC: Area under curve

IQR: Interquartile range

N: Number

p-value: Comparison of patients' salivary cortisol on hydrocortisone compared with healthy control subjects' endogenous cortisol

$p < 0.05$ considered significant

*$p < 0.05$

The greatest difference in median salivary cortisol between patients and controls were 23- and 25-fold at 09h00 and 09h30 respectively. The magnitude of the difference in the median cortisol levels between Addison's patients and healthy control subjects falls during the period from 14h00 until midnight, where the difference varies from almost 10- to 4.4-fold higher in the Addison's patients. Among patients and controls, the *p*-value was <0.0001 at each time point apart from 08h00, using repeated measures analysis of variance (ANOVA) test. The concentration of salivary cortisol also differed by the time point measured ($p < 0.0001$). In summary, Addison's patients are exposed to a higher median cortisol level throughout a 16-hour day curve, compared to control subjects.

6.4.2 Correlation between hydrocortisone dose and peak salivary cortisol concentrations

There was no correlation between hydrocortisone dose and peak salivary cortisol levels ($r = 0.18$; $p = 0.32$). The median salivary cortisol of the Addison's patients was significantly greater than that of the controls at each time point, with the exception of 08h00 (Figure 22). This is of relevance, since it is expected that the median cortisol level at 08h00 represents the basal concentration in patients with Addison's disease. Although the first sample was ostensibly taken prior to the usual dose of hydrocortisone in several patients, it was greater than the healthy subjects' endogenous salivary cortisol concentrations, suggesting that patients may exhibit residual endogenous cortisol concentrations or that patients may not have complied with the study requirement that their initial salivary cortisol be sampled immediately prior to their first usual dose of hydrocortisone.

6.4.3 Sampling time of salivary cortisol, hydrocortisone dose and correlation with AUC

It is expensive and cumbersome to take multiple salivary samples from individual patients on hydrocortisone replacement therapy. Therefore, should a single sample be highly correlated with AUC, it may in time serve as a surrogate marker

for AUC and consequently, be enough to indicate either adequate, under- or over-replacement. The time point that showed the best correlation with AUC was 08h30 (r = 0.67), representing C_{max}, which could potentially indicate the best surrogate marker for AUC. The most ideal time points to sample salivary cortisol concentrations in healthy control subjects appeared to be 08h00 and 08h30, by virtue of their strong correlation with AUC (Table 36). The scatterplots demonstrate a lack of correlation between total daily dose of hydrocortisone, hydrocortisone dose/kg, total dose of hydrocortisone/m^2 and AUC (Figure 23). The Spearman correlations were not significant and remained non-significant, even after adjusting for gender, age, race and foreign ancestry (Table 37).

Table 36: Time points reflecting sampling of salivary cortisol, showing the strongest correlations with AUC for healthy control subjects

Time Point	Correlation coefficient (r)	p-value
08h00	0.53	**0.003***
08h30	0.66	**0.0001***

AUC: Area under the curve

p-value: Correlation of salivary cortisol at time points with AUC

$p < 0.05$ considered significant

*$p < 0.05$

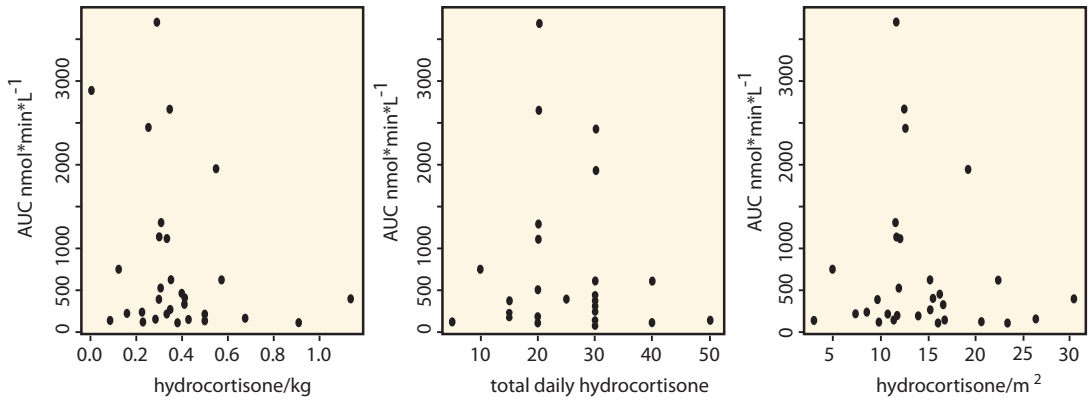

Figure 23: Scatterplots showing the relationship between AUC and hydrocortisone dose

The scatterplots above demonstrate a lack of correlation between AUC and dose of hydrocortisone, expressed as total daily hydrocortisone dose, total daily hydrocortisone dose/kg and hydrocortisone/m² (total daily hydrocortisone dose adjusted for body surface area)

Table 37: Correlation of hydrocortisone dose with salivary cortisol AUC (unadjusted) and adjusted for gender, age, race and foreign ancestry in 31 Addison's patients

	Spearman (unadjusted)		Adjusted
Dose	***r***	***p*-value**	***p*-value**
Total daily hydrocortisone dose	0.0336	0.86	0.77
Total daily hydrocortisone dose/kg	-0.2043	0.27	0.70
Total daily hydrocortisone dose/m²	-0.0567	0.77	0.92

Total daily hydrocortisone dose/kg: Total hydrocortisone dose per kilogram

Total hydrocortisone/m²: Total daily hydrocortisone dose per square metre of body surface area

r: Correlation coefficient

AUC: Area under the curve

$p < 0.05$ considered significant

6.4.4 Quantification of the variability of salivary cortisol in both patients and controls

As the sample was not normally distributed, the median value was used as a central measure of the analyte. The IQR was used to express the dispersion of the analyte in patients and controls. The dispersion of salivary cortisol was overtly greater in the Addison's patients compared to the controls (Table 38).

Table 38: Quantification of the variability of the salivary cortisol assay using interquartile ranges for each time point

Time Point	Median cortisol levels nmol/L (IQR) (Addison's patients, *N* = 31)	Median cortisol levels nmol/L (IQR) (Healthy control subjects, *N* = 30)
08h00	1.32 (0.72-9.94)	4.89 (2.95-4.89)
08h30	55.35 (21.39-534.93)	4.05 (2.28-5.22)
09h00	60.12 (26.5-130.68)	2.59 (1.56-4.69)
09h30	47.53 (26.60-101.48)	1.86 (0.93-3.03)
10h00	40.76 (27.15-66.7)	1.31 (0.72-3.00)
10h30	24.46 (19.0-67.90)	1.2 (0.62-2.41)
11h00	19.09 (11.23-35.86)	0.81 (0.50 -1.69)
12h00	12.09 (7.66-20.55)	0.80 (0.50 -1.34)
14h00	7.07 (2.22-13.76)	1.24 (0.74-2.03)
16h00	4.64 (1.69-12.85)	0.58 (0.50 -1.42)
16h30	4.91 (2.56-11.84)	0.50 (0.50 -1.10)
17h30	4.37 (1.82-12.57)	0.50 (0.50 -0.96)
19h00	2.68 (0.69-4.45)	0.50 (0.50 -0.62)
21h00	2.2 (0.85-4.19)	0.50 (0.50 -0.78)
22h00	2.92 (0.89-4.4)	0.50 (0.50 -0.63)
00h00	2.17 (0.49-6.16)	0.50 (0.50 -0.50)

N: Number

IQR: Interquartile ranges

Significant variability is detected at each time point using interquartile ranges, but it is exaggerated among the patients, particularly in the morning time points. Substantially less variability is noted in the evening hours for both patients and controls, with controls exhibiting the least variability from 17h30–00h00.

0.05 nmol/L represents the lower limit of detection.

In the individual timed salivary cortisol concentration plots for the patients on hydrocortisone replacement, there was considerable variability (Figure 24). Despite the fact that the largest dose of hydrocortisone was taken immediately after the 08h00 sample, the highest saliva cortisol peak is not invariably observed in the first part of the day. The salivary cortisol concentrations at 08h00 for each of the patients do not uniformly represent the nadir. In addition, several patients demonstrated incomplete salivary cortisol day curve data, for example, subject numbers 8, 16 and 30.

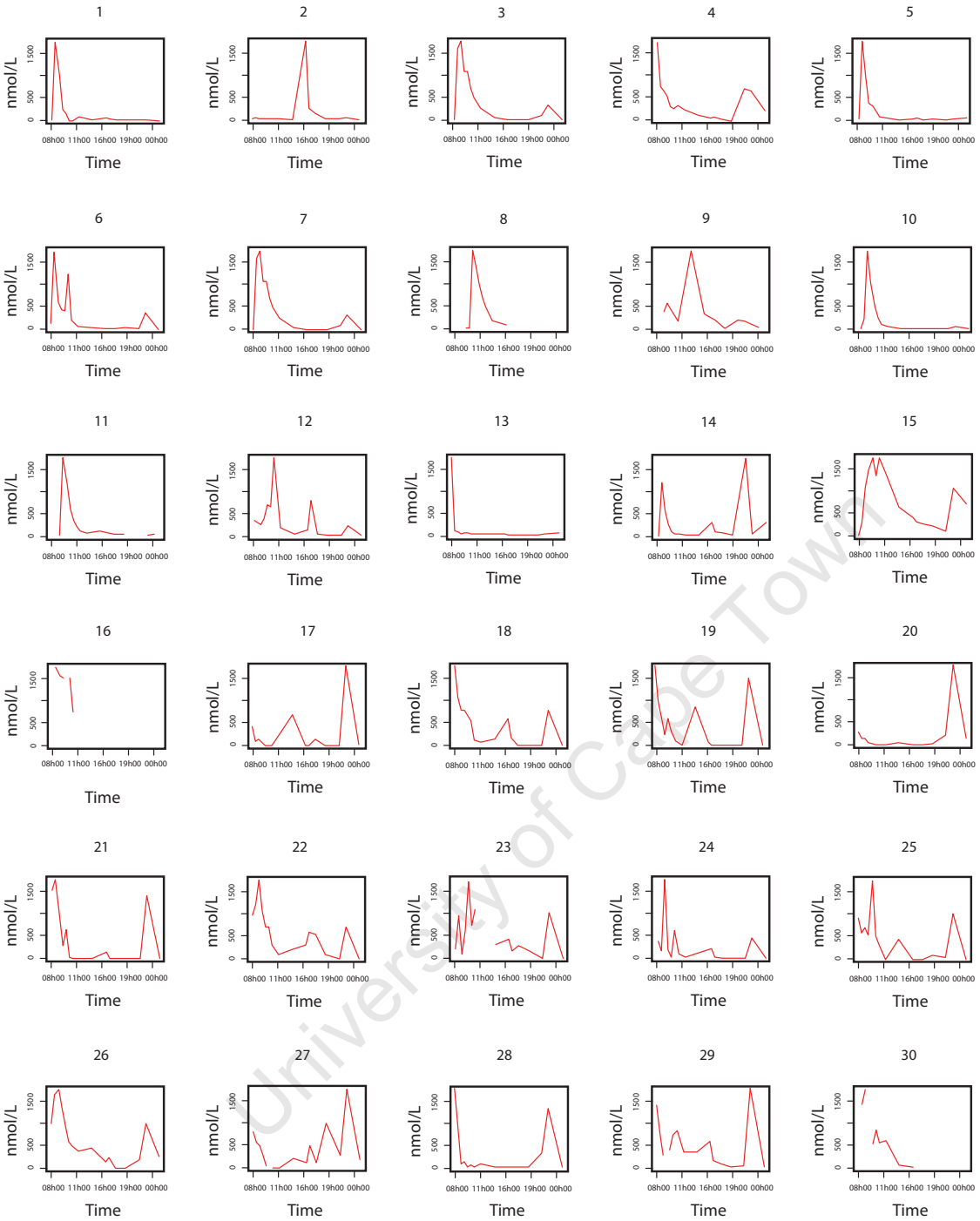

Figure 24: The individual timed salivary cortisol concentration plots for each of the patients. There is a lack of uniformity for each of the salivary cortisol concentration plots. Considerable variability is also observed. Despite the fact that the largest dose of hydrocortisone was taken immediately after the 08h00 sample, the highest salivary cortisol peak is not invariably observed in the first part of the day. The salivary cortisol concentration at 08h00 do not uniformly represent the nadir. Several patients demonstrated incomplete data, for example, subject numbers 8, 16 and 30.

6.4.5 Examination of the salivary cortisol reference range

The National Health Services Laboratory (NHLS) at Groote Schuur hospital set up the salivary cortisol assay using the Elecsys (Roche) system. The endogenous cortisol concentrations from 30 normal healthy subjects were evaluated. The purpose of this was to compare the results with those from Addison's patients on their usual hydrocortisone replacement therapy.

Using the package insert supplied by the manufacturer of the Elecsys (Roche) automated analyser, the proportions of patients and controls whose salivary cortisol was either lower than or in excess of the reference ranges, were calculated (Table 39). For the afternoon, the manufacturer had supplied reference ranges for the time interval between 14h30 and 15h30, but it was deemed that since sampling in this study occurred at 14h00 and 16h00, these time points were sufficiently close for comparison. Interestingly, none of the healthy controls exhibited a salivary cortisol above the reference range supplied by the manufacturer. As many as 73% at the 14h00 time point demonstrated a salivary cortisol below the reference range, in contrast, a substantial proportion exhibited a salivary cortisol below the reference range for the predetermined time points. However, the majority of patients exhibited salivary cortisol concentrations above the reference range for each of the time points and only a minority of patients demonstrated a salivary cortisol below the reference range, except at the 08h00 time point. The manufacturer's reference range is derived from the 5th to the 95th percentile, so within this reference range, it would be expected that 90% of the controls would exhibit salivary cortisol in this range. As none of the control subjects exhibited salivary cortisol concentrations above the reference range, and all outliers were below the reference range, it may indicate that the reference range was too high for the population sampled in this study. Therefore, the manufacturer's reference range could not be validated for these healthy control subjects and so the reference range supplied by the manufacturer, by inference, is not transferable. It should be stated that the collection technique used was not recommended by the manufacturer,

as it included an additional step of instilling saliva from a microcentrifuge onto a salivette, which could account for the substantial proportion of healthy controls who exhibited salivary cortisol levels lower than the recommended lower limit of the reference range.

The fact that there were very few samples from healthy controls and that they were not normally distributed, represented an obstacle in determining a reference range in the study. Ideally, a minimum of 120 samples are needed to determine the 5th to the 95th percentile in a non-parametric data set. Nevertheless, the CI was calculated for the 5th and the 95th percentiles at each time point. For example, at 08h00, the lower reference range was 0.96 nmol/L, with a CI of 0.79-1.01 nmol/L, whereas the upper reference range was 10.47 nmol/L with a wide CI of 8.58-15.27 nmol/L using the 90% confidence intervals for each value suggesting uncertainty as to the true ranges. At midnight for example, the upper limit appeared to be 2.35 nmol/L with a CI of 1.12-4.62 nmol/L. A lower reference range for midnight could not be determined because 0.50 nmol/L probably represents the limit of detection for the assay. The 90% CI is utilised by convention to express the certainty of the 95% reference range. In view of the small numbers, these limits are unlikely to be reliable. Notably, there are discrepancies between the manufacturer's reference range and the healthy ranges, suggesting that the former may not be applicable in this healthy population. It was also confirmed that the Addison's patients exhibited higher ranges of salivary cortisol compared to the healthy control subjects.

Table 39: Proportion of Addison's disease patients and controls, whose salivary cortisol levels fell outside the recommended reference range, using the Elecsys (Roche) assay

Time point	Reference range	Proportion of patients *n/N* (%)	Proportion of control subjects *n/N* (%)	*p*-value Patients versus controls
08h00 < 1.9 nmol/L > 19.0 nmol/L	1.9-19.0 nmol/L	 15/30 (50) 5/30 (17)	 5/29 (17) 0/29 (0)	 **0.01*** 0.06
08h30 < 1.9 nmol/L > 19.0 nmol/L	1.9-19.0 nmol/L	 2/30 (8) 23/30 (77)	 3/30 (10) 0/30 (0)	 1.0 **0.0001***
09h00 < 1.9 nmol/L > 19.0 nmol/L	1.9-19.0 nmol/L	 1/30 (3) 25/30 (83)	 7/30 (23) 0/30 (0)	 0.06 **0.0001***
09h30 < 1.9 nmol/L > 19.0 nmol/L	1.9-19.0 nmol/L	 0/31 (0) 25/30 (83)	 14/30 (47) 0/30 (0)	 **0.0001*** **0.0001***
10h00 < 1.9 nmol/L > 19.0 nmol/L	1.9-19.0 nmol/L	 0/30 (0) 26/30 (87)	 18/30 (60) 0/30 (0)	 **0.0001*** **0.0001***
14h00 < 2.05 nmol/L > 11.9 nmol/L	2.05-11.9 nmol/L	 7/30 (23) 10/30 (33)	 22/30 (73) 0/30 (0)	 **0.001*** **0.001***
16h00 < 2.05 nmol/L > 11.9 nmol/L	2.05-11.9 nmol/L	 8/29 (28) 8/29 (28)	 25/30 (83) 0/30 (0)	 **0.0001*** **0.007***

n: numbers of patients and controls identified with salivary cortisol outside the reference range

N: Total number of patients and control subjects

p-value patients versus controls

$p < 0.05$ considered significant

$^{*}p < 0.05$

6.4.6 Lipids, lipoproteins and biochemical markers of cardiovascular inflammation in the salivary cortisol sub-study, and the remaining cohort of Addison's patients

The median levels of TSH, RBG, TC, TG, HDLC, LDLC, NEFA and hs-CRP were comparable in both the salivary cortisol study and the remainder of the Addison's disease cohort. No association was identified between AUC and any of the aforementioned metabolic characteristics.

6.5 Discussion

The principal finding of this study was that Addison's disease patients, irrespective of the dose of hydrocortisone used, had a significantly greater exposure to cortisol than healthy control subjects, when assessed using AUC or peak salivary cortisol measurements. Additionally, the degree of salivary cortisol exposure, as shown by AUC, did not correlate with measured metabolic abnormalities.

Although this study sample size is small, the differences in salivary cortisol levels between healthy control subjects and patients were large, and there is less than 1% likelihood that these differences were due to chance alone. This study is unique in many respects. First, it examines salivary cortisol in one of the largest exclusively primary hypoadrenal cohorts. Many other similar studies included patients with both pituitary and adrenal disease. Second, in contrast to previous studies, it has generated credible evidence for excess exposure using AUC, with what is believed to be physiological replacement of hydrocortisone therapy. Third, apart from the report by Lovas et al, this study of South African Addison's disease patients' salivary cortisol, which analysed a day curve over 16 hours with multiple sampling, is considerably longer than many others.[9] Fourth, it confirms that an intensive survey of cortisol excursions in the comfort of patients' homes can be accomplished, even in rural and isolated regions of a vast country where infrastructure is poor.

The patients who participated in the salivary cortisol study differed from the entire Addison's disease cohort in terms of ethnicity and differed from healthy control subjects in terms of ethnicity, gender and age. Nevertheless, it is speculated that most of the findings can be generalisable to the entire cohort. Although cortisol production rates are higher in men compared to women,[24] when using steady-state stable isotope tracer infusion deuterated cortisol, this was no longer significant when adjusted for body surface area. Additionally, increased metabolites of cortisol have been found in men compared to women, which may offset the higher cortisol production rates.[25] Moreover, black and white women did not differ in the evaluation of the HPA axis using the 24-hour urinary excretion of cortisol and dexamethasone suppressability, matched for age, weight, BMI and body surface area.[26] Despite some literature indicating differences in cortisol production between genders and among various ethnic groups, it is likely that the findings from those enrolled in the salivary cortisol study are applicable to the remaining group of Addison's patients. The vast differences observed in the salivary cortisol excursions between patients and healthy control subjects are not likely due to these factors.

This study has clearly demonstrated that exposure to cortisol is significantly greater in patients with Addison's disease on hydrocortisone therapy compared to endogenous cortisol secretion in healthy control subjects. This was shown by the median AUC, which was 15-fold greater in magnitude than that seen in healthy control subjects. Only one other study used salivary cortisol AUC as a measure of hydrocortisone exposure in Addison's disease and hypopituitarism. The study's purpose was to examine the correlation between saliva and plasma exposure, following hydrocortisone administered both intravenously and orally in hypoadrenal patients. Although the correlation was poor, it was not designed to compare exposure of cortisol in hypoadrenalism on replacement therapy to endogenous cortisol exposure in healthy subjects.[27]

The vast difference in AUC is likely to be true as possible causes for spuriously elevated cortisol levels were minimised. Drugs and interfering substances may cause spurious elevations of salivary cortisol. However, drugs that potentially could raise salivary cortisol concentrations were exclusion criteria in this study. As cross-reactivity of cortisol and cortisone is known to occur in some automated assays, the cross-reactivity with cortisone, using the Elecsys (Roche) analyser, is limited to 0.3% at 74.5 nmol/L of cortisol and so it represents an attractive assay methodology.[14] Several AUC levels were very high and the sample size was small, none of the outliers was eliminated from the analysis. If the very high levels were removed, it could have reduced the sample size considerably, but the large differences would remain. A number of conditions are associated with elevated salivary cortisol levels. Salivary cortisol was found to be elevated in patients with anorexia nervosa, whether treated or untreated, but not in patients with obesity compared to controls.[28] As none of the patients in the current study was anorexic, this factor could not account for the remarkably elevated AUC levels. The role of the fasting or fed state on AUC is unclear. Aanderud et al showed that AUC was greater if patients had breakfast prior to their cortisone dose compared to taking it after an overnight fast, while Barbhaiya et al reported that cortisol levels were lower after eating.[29–32] Johannsson et al, utilising a modified-release hydrocortisone tablet in healthy volunteers, demonstrated a significantly greater plasma cortisol AUC in the fed state compared to the fasting state, and this finding suggests that food accelerates the solubility and the dissolution rate in the gastrointestinal system.[33] In this study of Addison's patients, it is uncertain whether sampling the cortisol levels in a fasting state would have significantly altered the outcome of the AUC levels.

The body of evidence, with respect to hydrocortisone replacement therapy in Addison's disease and hypopituitarism, points to the supra-physiological peaks of cortisol following ingestion, followed by the inexorable decline of cortisol levels.[6 8 34] The median salivary peak cortisol, C_{max}, found by Thomson et al, was

150 nmol/L (t_{max} 50 minutes) following usual oral hydrocortisone doses,[27] which was similar to the 174.5 nmol/L (t_{max} 60 min) found in this study. Among patients with anorexia nervosa, the total AUC for plasma cortisol was approximately 1.5 times greater than that of healthy controls.[35] In alcohol-dependent persons who are going through alcohol withdrawal, the mean daily salivary cortisol levels, as calculated by AUC, was 2.56 nmol*min*L^{-1} compared to control levels of 0.5 nmol*min*L^{-1}.[36] Similarly, the AUC for patients with chronic fatigue syndrome was 2.3 nmol*min*L^{-1}.[37] Taken together, the AUC for salivary cortisol determined in Addison's disease patients using hydrocortisone is considerably greater than in patients with anorexia nervosa and alcohol withdrawal. On the other hand, chronic fatigue syndrome appears to induce lower AUC for salivary cortisol than controls,[37] and it is therefore expected that Addison's patients have higher AUC for salivary cortisol than chronic fatigue syndrome sufferers.

Some studies of salivary cortisol in Addison's disease have confirmed a high degree of correlation between plasma and salivary cortisol,[2 3 10] while Wong et al showed a poor correlation attributed to the rapid changes of free cortisol in plasma, which occur soon after administration of hydrocortisone. These levels rise sharply due to saturation of CBG. It raises some uncertainty as to whether the serum cortisol AUC in these Addison's patients was also elevated.[10] The significantly elevated AUC for salivary cortisol, and thus increased exposure to GCs, may translate into unwanted side-effects. Specifically among individuals aged between 50–70 years of age, the higher AUC for salivary cortisol was associated with impairment of cognitive function.[38] The endogenous total salivary cortisol exposure, as measured by AUC, was found to be positively correlated with the number of plaques in the carotid arteries, having been adjusted for associated CV risk and socio-demographic factors in an elderly population group.[39] It was suggested that the accelerated atherosclerosis resulted from the propensity of cortisol to induce diabetes, hypertension and lipid derangements.[30 39] Furlan et al reported positive and negative associations respectively between salivary cortisol

AUC, depression and BMD.[40] The impaired quality of life and feelings of fatigue identified in patients with Addison's disease have been attributed to the low levels of cortisol during the day and late evening, but it is equally conceivable, since high cortisol concentrations are associated with depression, that this symptom may be accounted for by the high salivary cortisol AUC seen in this cohort of the South African Addison's disease patients. Moreover, a link has been demonstrated between elevations of salivary cortisol AUC and diabetes mellitus among Jewish Immigrants.[41]

The only adverse effects examined in this small study were those of lipids, lipoproteins and markers of CV inflammation. It is not surprising that there is a lack of association between salivary cortisol exposure and metabolic derangement, since the study size limited its power. Salivary cortisol levels were highly variable, and although the salivary cortisol was sampled frequently during the day curve in this study, the finding of increased salivary cortisol may fail to reflect that which transpires on a cellular level. Lipid and lipoprotein values in a given individual are the result of multiple co-existing metabolic processes, and only a few endpoints have been examined in the current study. The effects of, inter alia, ageing, menopause, personal diets and genetic factors were not considered in the evaluation of this cohort.[42–45] It is also entirely plausible that patients may have individual sensitivities to cortisol. Previous work has confirmed the presence of multiple GCR polymorphisms, which may have a role to play, whether patients manifest with metabolic derangements or not.[46–48] These receptors are known to induce either enhanced or reduced sensitivity, and the finding of either a high or a low AUC may fail to reflect the post-receptor exposure to cortisol. Thus, a degree of cortisol receptor resistance may be protective in those individuals with excessive AUC for cortisol. These GCR polymorphisms are known to occur sporadically and are unlikely, on their own, to account for the mismatch between high cortisol exposure levels and failure to demonstrate metabolic changes. Indeed, work by Morton et al showed that cortisol levels in patients with simple obesity may be

only modestly elevated and this has been ascribed to alterations in the enzyme 11 beta-hydroxysteroid-dehydrogenases, which may alter GC action on fat tissue either by enhancing its sensitivity or by reducing its efficacy at tissue level.[49] Alterations in the kinetics of the enzyme 11 beta-hydroxysteroid-dehydrogenase were not examined in this cohort, but may account for the reduced sensitivity seen in relation to salivary cortisol concentrations and consequent absence of metabolic derangements. It is most likely that a large sample will finally resolve whether a relationship exists between AUC for salivary cortisol and metabolic derangements.

A considerable degree of inter-individual variability in salivary cortisol concentration was noted in both the patients and controls, as determined by the wide IQR to express the dispersion of the analyte. It is conceivable that factors relating to sample collection may have played a role in this degree of inter-individual variability. This variability has previously been documented.[6 10 29] These factors include the possibility of mislabelled tubes, incorrect recording times and contamination of saliva with the hydrocortisone tablets. Additionally, one cannot ignore the heterogeneity of the group with respect to age range and that the cause of Addison's disease, as well as unknown factors, may be contributing to this variability, for example, gastro-intestinal factors.[50] The additional step, not specifically recommended by the manufacturer, of instilling saliva on to salivettes following passive drool collection, could have also contributed to the variability.

The median salivary cortisol of Addison's patients was significantly greater than in controls at each time point, with the exception of 08h00. This is relevant since the patients were requested to take their morning hydrocortisone dose immediately after collecting the 08h00 sample. Smans et al reported partial recovery of cortisol secretion in a patient with autoimmune adrenalitis having been observed for 7 years and deciding on his own volition to stop replacement therapy.[51] Other authors have reported similar events,[52] and in 1987, it was reported that Addison's disease

caused by lymphomatous infiltration could be reversed following successful chemotherapy.[53] It is not clear whether the phenomenon of partial recovery may account for the higher than expected basal cortisol levels found in the present study, or alternatively that erroneous sampling of salivary cortisol, following the hydrocortisone dose instead of immediately prior to it, may play a role.

In many cases, the healthy control subjects' salivary cortisol did not fall within the manufacturer's reference range; especially as a significant proportion had salivary cortisol levels below the reference range. It was therefore deemed that this reference range was not transferable to this dataset. Since the salivary cortisols of the healthy controls exhibited a non-normal distribution and were too few in number, it was appreciated that a reference range from this data could not be generated. The theoretical minimum sample size required for the estimation of the 100α and 100 (1 – α) percentiles is equal to 1/α. The estimation of the 2.5 percentile requires at least 1/0.025 = 40 observations. The precision of percentiles increases with increasing numbers of observations and a reduction of CI. It has been suggested that a minimum of 120 reference values are required and even more are necessary when the distribution of data is non-normal.[54 55]

The observation that the 08h30 time point for sampling salivary cortisol correlated well with the AUC, suggests that this may serve as a surrogate marker for AUC and peak cortisol levels. It may in time serve as a useful indicator of either under- or over-replacement with hydrocortisone therapy. On the other hand, Mah et al found that the 4-hour single plasma measurement after the initial hydrocortisone dose correlated reliably with the AUC.[29] The difference is probably due to the study being performed using plasma cortisol, which may take time to equilibrate with CBG, unlike salivary cortisol, which is an instantaneous measure of free cortisol. Indeed, salivary cortisol may have a specific advantage in its correlation with AUC, as it can be sampled half an hour after hydrocortisone ingestion, provided that the required precautions are taken. Arlt et al suggested that the plasma

cortisol taken 120 min and 360 min after the morning dose would be the best samples to determine whether patients are either under- or over-treated by virtue of the cortisol excursions following replacement therapy.[56] However, this should ideally be used in conjunction with the clinical features to confirm either over- or under-replacement. While plasma cortisol has been used to assess adequacy of replacement, salivary cortisol may in time gain the scientific credence for routine use.

As the cortisol exposure appears to be markedly supra-physiological in patients on hydrocortisone replacement therapy, it begs the question of whether these patients require the hydrocortisone replacement to be escalated during times of stress. Despite the high observed concentrations of cortisol, escalating therapy during stressful conditions is still advised until further research demonstrates the contrary.

In the preliminary studies of salivary cortisol, a poor correlation was obtained from saliva collected by passive drool into microcentrifuge tubes and salivettes (r = 0.37). Subsequent analysis showed that a sub-optimal correlation of r = 0.96 (r = 0.975 is considered a perfect correlation) exists between salivary cortisol collected in microcentrifuge tubes and then being instilled onto salivettes compared to salivettes alone. Although this relationship is acknowledged as sub-optimal for method comparisons, it was considered adequate for general comparisons.[55] All salivary cortisol samples were collected by initial passive drool into microcentrifuge tubes throughout the study and were subsequently instilled onto salivettes.

6.5.1 Weaknesses of this study

This study has a number of weaknesses. A significant degree of inter-individual variability in salivary cortisol levels was noted. Although this is well described, the samples were not collected under supervision, with the possibility that

utilisation of mislabelled tubes or eating at the incorrect times could not be excluded. The addition of a pre-analytical step could have contributed to the higher than expected concentrations seen in Addison's patients. The sample size was small and so limited the power of the study to examine the relationships with metabolic parameters. It would have been preferable to perform salivary analyses in hospital. On the other hand, the widespread geographical distribution of the patients precluded admission to hospital. It is acknowledged that choosing medical students as a control population may have inherent problems as they have likely a higher degree of compliance to a medial study. Moreover as the first salivary cortisol sample was taken at 08h00, it is conceivable that the peak could have been missed if it were earlier.

6.5.2 Conclusions and future recommendations

A significantly elevated cortisol exposure has been confirmed in the Addison's patients. This may represent entirely artifactually high concentrations with no deleterious effects, or, it is more likely that the inordinately high concentrations may be directly harmful. It is conceivable that salivary cortisol reflects tissue exposure much more closely than serum cortisol, since the former is free cortisol.

Potentially, the 30 minute salivary sample taken after hydrocortisone ingestion is a likely candidate as a surrogate marker for AUC and this should be confirmed in further studies. A future line of investigation would be to examine the correlation between AUC for salivary cortisol and metabolic parameters, especially lipids and markers of bone turnover in considerably larger samples. Once this has been accomplished and the association with metabolic consequences excluded, only then can we confidently deduce that current forms of routine replacement in Addison's disease are not rendering significant harm.

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Chapter 7
The effect of glucocorticoid receptor polymorphisms on the sensitivity to cortisol in Addison's disease

7.1 Introduction

Addison's patients require lifelong hydrocortisone replacement therapy, but there is uncertainty as to the ideal dose of hydrocortisone. Despite the impression that patients are adequately supplemented, many Addison's patients report subjectively impaired health quality.[1] Clinicians often respond by raising the doses of hydrocortisone in an attempt to reverse this subjective health impairment with potentially deleterious effects on multiple systems.[1–6] It has not yet been established whether the presence of specific GCR alleles induce a degree of GC resistance, necessitating increased doses of hydrocortisone. On the other hand, if deleterious metabolic consequences are identified in association with sensitising GCR polymorphisms, it may provide impetus to modify the dose of hydrocortisone in accordance with the specific GCR polymorphism.

There is a dearth of information as to whether GCR polymorphisms play a role in the development of GC-related side-effects in individuals receiving hydrocortisone replacement for Addison's disease. In addition, there is uncertainty as to whether the empiric dose of hydrocortisone needs to be altered in the presence of certain GCR polymorphisms.

7.2 Aims

The purpose of this study is to determine whether metabolic alterations are associated with GCR polymorphisms sufficiently to warrant modifications of

hydrocortisone replacement. This so far has been empiric.

The objectives of this study are to:

i. explore the role of GCR polymorphisms in influencing metabolic parameters among patients with Addison's disease by comparing or correlating GCR genotypes with clinical parameters including BMI, TC, TG, HDLC, LDLC, hs-CRP, NEFA, small dense LDL, TSH and hydrocortisone dose
ii. determine whether clinical differences exist among patients who harbour GCR polymorphisms with either increased or decreased sensitivity compared to those with wild type.

7.3 Patients and methods

One hundred and forty seven (n = 147) patients with Addison's disease were age, gender, ethnicity and BMI matched with 147 control subjects who attended a blood donor clinic. As there were limited numbers of healthy control subjects available, their selection was based on the closest possible match with the Addison's disease patients. As a generalisation, there were far more potential white healthy control subjects available compared with other ethnic groups. Thus, the white healthy control subjects could be selected in a ratio of 1:10, whereas the mixed ancestry controls could only be selected in a ratio of 1:4 basis and for the black controls, 11 were selected from a possible 15 subjects. White and mixed ancestry patients were far better matched to their respective controls compared to the black and Asian patients. These latter two ethnic groups were represented by too few healthy volunteers and overall the matching was imperfect. The majority of the South African Addison's disease cohort (95%) was enrolled in this study after signing written informed consent.

7.3.1 Genotyping for glucocorticoid receptor polymorphisms

In this section the methods for DNA extraction and detection of GCR polymorphisms of interest are described.

7.3.1.1 DNA extraction

Genomic DNA purification was performed using a Promega Wizard Kit from Wisconsin, USA. 900 µl of cell lysis buffer was added to a 1.5-ml eppendorf tube, to which 300 µl of blood had been previously added. Once the tube had been inverted five to six times to mix the solution, it was left standing at room temperature for 10 minutes, during which time it was inverted twice. The entire mixture was centrifuged at 14 000 centrifugal accelerations related to gravity (xg) for 1 minute in a microcentrifuge tube. The supernatant was then poured off, leaving a small amount of residual liquid. Excess liquid was removed by blotting the inverted tube onto tissue. If the pellet was very red, 300 µl of lysis buffer was added and further lysis buffer was added. The pellet was resuspended by vigorously vortexing it in 300 µl of nuclei lysis solution. Mixing was performed by sucking the suspension up and down, using a 1-ml Gilson pipette. 100 µl of protein precipitation solution was added, which was followed by a 15–20 seconds of vigorous vortex. This was followed by 14 000 xg of centrifuging for 3.5 minutes at room temperature. The supernatant was then decanted and the remaining liquid was blotted onto tissue. The pellet was then washed with 300 µl of 70% ethanol. This was inverted to mix the solution and followed by 3 minutes of further centrifuging, after which the supernatant was discarded. The tube was then drained upside down onto tissue for 30 minutes and 100 µl of DNA rehydrating solution was added and incubated overnight at room temperature. The DNA was stored at 4 °C for hours and at –20°C for longer periods.

7.3.1.2 Detection of the GCR single nucleotide polymorphisms: BclI, E22/23EK and N363S

The genotyping of the GCR gene for detecting the single nucleotide changes *Bcl*I G/C (rs41423247), ER22/23EK AA/GG (rs6189) and N363S G/A (rs6195) was performed by PCR amplication, followed by restriction length fragment length analysis. The primer pairs used in the PCR amplification were 5'-GCA GTG AAC AGT GGT ACC AGA CC-3' / 5'-AAA GAG AAA AAT CAA ACG AAA GC-3', 5'-GCT

GCC TCT TAC TAA TCG GAT CA-3' / 5'-TTT AAG TCT GTT TCC CCC GAG-3' and 5'-TTT AAT GTC ATT CCA CCT ATT CCC-3' / 5'-AGC CAT TAG AAA AAA CTG TTC GAC-3' respectively. The primers were designed by using Oligo.exe version 3.4 (National Bio-sciences, Hamel, Minnesota USA) (Table 40). Each PCR contained approximately 100–500 ng/µl genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200µM of deoxyribonucleotide triphosphate (dNTP), 15 pM of each primer and 0.75U Thermus aquaticus (Taq) DNA polymerase (Promega–Go Taq Flexi DNA Polymerase) in a final volume of 30 µl. The PCR conditions for all reactions were the same. The denaturation step was performed at 98 °C for 2 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds and annealing was carried out at 52 °C for 30 seconds. Elongation occurred at 72 °C for 30 seconds and a final extension occurred at 72 °C for 8 minutes. The rs41423247 PCR products were digested using *Bcl*I restriction enzyme (C allele = 146 + 86 base pairs (bp), G allele = 232 bp), rs6189 PCR products were digested using Mn1 restriction enzyme (GAG AGG allele = 201 + 143 + 50 + 35 + 15 + 11 + 3 bp, GAA AAG allele = 201 + 178 + 50 + 15 + 11 + 3 bp) and rs6195 PCR products were digested, using Tsp 5091 (A allele 91 + 36 bp, G allele = 127 bp), according to manufacturers' instructions. The products were separated on an agarose gel, visualised with ethidium bromide and the sizes of the fragments were compared to the standard marker (1 kB plus (Promega®)) (Figure 25).

Table 40: Primer pairs used for the detection of single nucleotide polymorphisms (SNPs) of the GCR

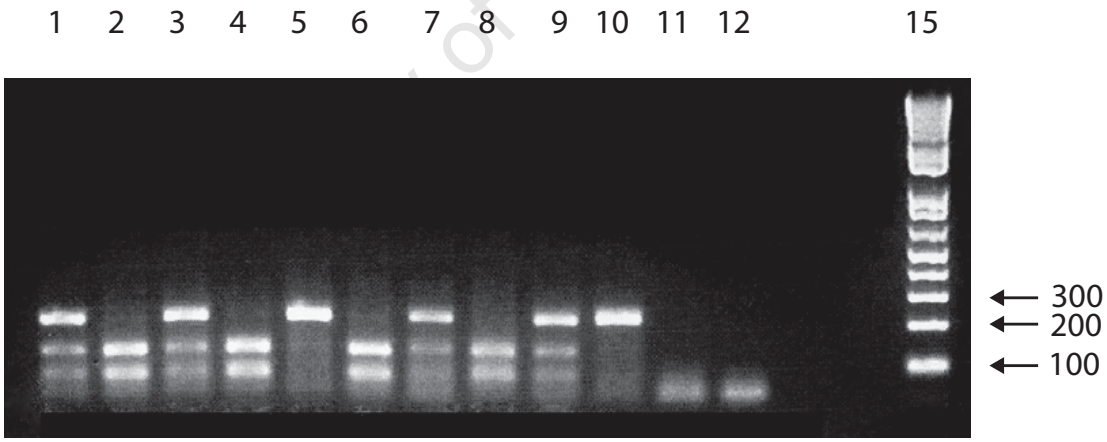
Polymorphism	SNP	Forward primer Reverse primer
*Bcl*I G/C	rs41423247	5'-GCA GTG AAC AGT GGT ACC AGA CC-3' 5'-AAA GAG AAA AAT CAA ACG AAA GC-3
ER22/23EK AA/GG	rs6189	5'-GCT GCC TCT TAC TAA TCG GAT CA-3' 5'-TTT AAG TCT GTT TCC CCC GAG-3'
N363S G/A	rs6195	5'-TTT AAT GTC ATT CCA CCT ATT CCC- 3' 5'-AGC CAT TAG AAA AAA CTG TTC GAC-3'

GCR: Glucocorticoid receptor

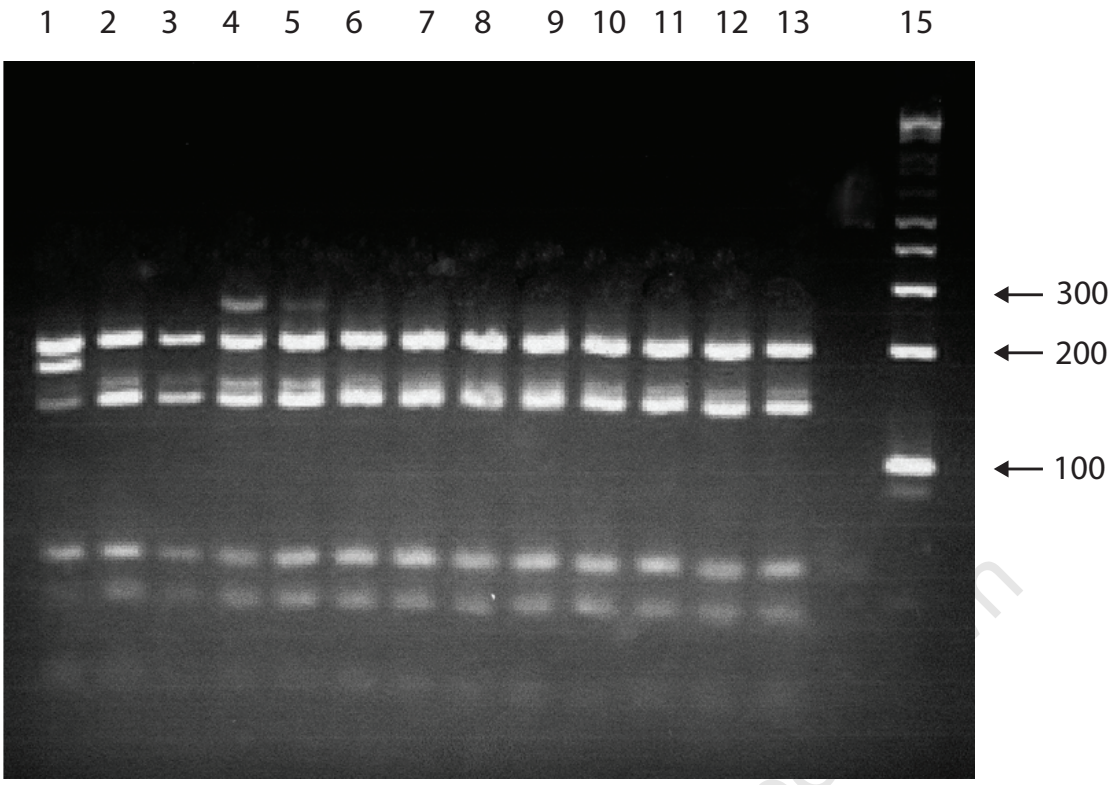
SNP: Single nucleotide polymorphism

rs: Reference SNP

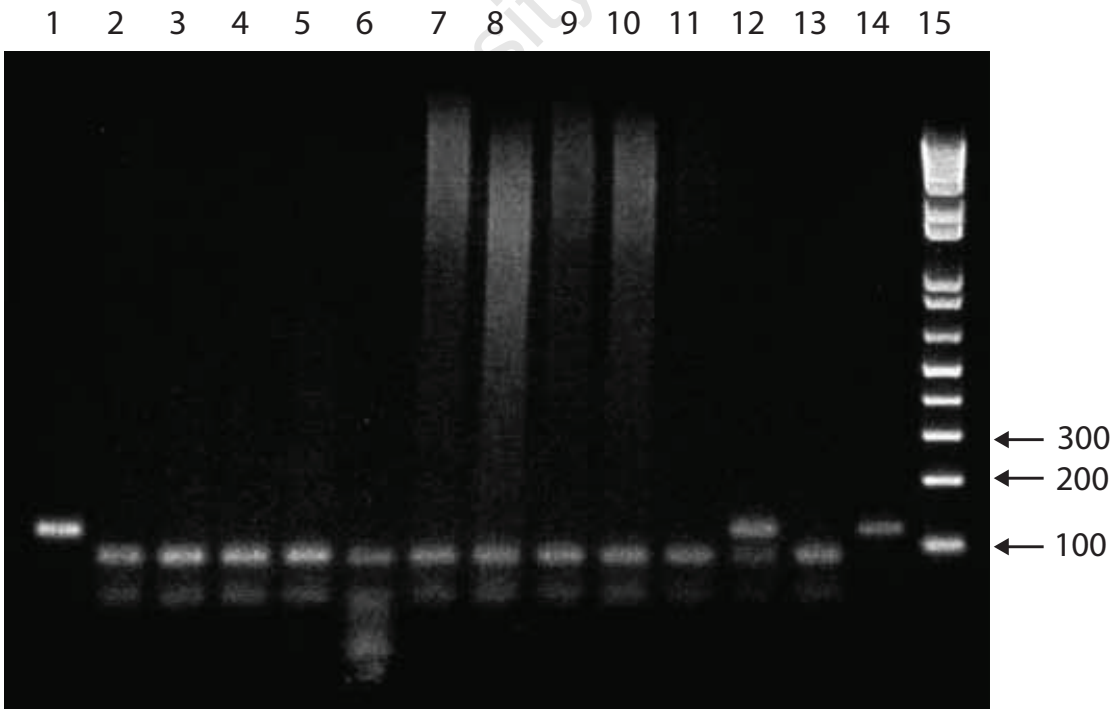
A, C, G, T = DNA bases



*Bcl*I Restriction enzyme digest was separated on 2% agarose gel, lane 1 heterozygous, lane 2 wild type, lane 3 heterozygous, lane 4 wild type, lane 5 homozygous, lane 6 wild type, lane 7 heterozygous, lane 8 wild type, lane 9 heterozygous, lane 10 homozygous, lanes 11 and 12 negative control, lane 15 1KB plus marker. (Wild type CC alleles; heterozygous CG alleles; homozygous GG alleles)



ER22/23EK polymorphism (rs6189) Restriction enzyme digest was separated on 4% agarose gel lane 1 heterozygous, lanes 2-13 wild type, lane 15 1KB plus marker (wild type GAG AGG/ GAG AGG, heterozygous GAG AGG/GAA AAG alleles)



N363S polymorphism (rs6195) Restriction enzyme digest was separated on a 2% agarose gel. lane 1 homozygous (positive control), lanes 2-11 wild type, lane 12 heterozygous, lane 13

wild type, lane 14 positive control, 15 1KB plus marker. (wild type AA allele, heterozygous AG, homozygous GG allele)

Figure 25: Agarose gel pictures showing digestion patterns for the three glucocorticoid receptor (GCR) polymorphisms

The genotyping quality was verified using the Hardy Weinberg Equilibrium (HWE) calculation on the controls and it was observed that none of the genotypes deviated from HWE, as shown by their respective *p*-values in parentheses: *Bcl*I (0.113), N363S (1.00) and ER22/23EK (1.00). None of the samples obtained was sequenced. The HWE test was also performed in ethnic sub-groups of healthy control subjects to determine whether there were problems with the genotyping methods. In the groups with sufficient sample size, the genotypes were also in HWE, suggesting that no problems were encountered with the genotyping methodology employed (Table 41).

Table 41: Hardy Weinberg equilibrium (HWE) test for controls (*p*-value), examined in relation to ethnicity

Polymorphism	White	Mixed Ancestry	Asian	Black
*Bcl*I (C to G allele)	0.45	0.56	0.709	_____*
ER22/23EK (GAG AGG to GAA AAG allele)	0.67	0.93	_____*	0.88
N363S (A to G allele)	0.75	0.93	_____*	_____*

In the groups with sufficient sample size, the genotypes were in Hardy Weinberg equilibrium, suggesting no problems with the genotyping methods.

$p < 0.05$ is considered significant

*: Groups with insufficient sample size

7.3.2 Statistical methods

Clinical characteristics of patients and controls were compared, regardless of ethnicity. Nonparametric and parametric data were compared, using the Mann-Whitney and student-T tests, respectively. The prevalences of polymorphisms between patients and their respective controls were compared. Mixed-effects logistic regression was used to compare the genotype distributions between patients and controls, while simultaneously controlling for ethnicity and matched pairs. Samples were analysed to determine whether the genotype had an effect on phenotype in healthy control subjects. Analysis was also undertaken to determine whether the genotype had an effect on phenotype in the Addison's patients. Further analysis was undertaken to determine whether the effect of the polymorphisms differed between patients and controls (interaction). These analyses were carried out using logistic mixed regression models, adjusting for BMI, gender, age and ethnicity in both patients and controls, while in Addison's patients foreign ancestry and hydrocortisone/m^2 were additionally adjusted for. For all analyses a *p*-value <0.05 was interpreted as indicating statistical significance.

7.4 Results

The pattern of enrolment of the 147 Addison's disease patients who participated in this study is shown in Figure 26.

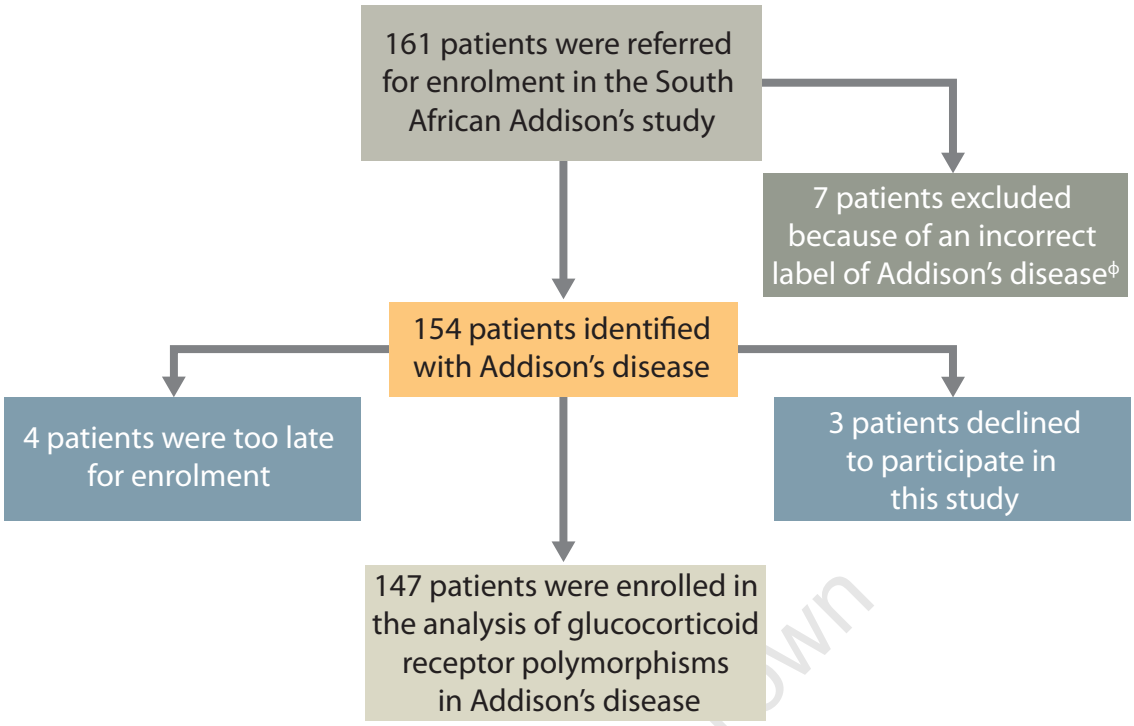


Figure 26: Pattern of enrolment of patients in the study of glucocorticoid receptor polymorphisms. [Φ]There were seven patients who were not included in the study because of an incorrect label of Addison's disease as two had normal ACTH stimulation test, two had secondary hypoadrenalism, one had bilateral adrenalectomy for Cushing's disease and two had suppression of the hypothalamic-pituitary adrenal (HPA) axis related to previous steroid use for another indication. Three patients declined to participate in this study citing personal reasons, and four were enrolled after DNA was analysed for glucocorticoid receptor polymorphisms. There were 147 patients who were ultimately enrolled in the study of glucocorticoid receptor polymorphisms in Addison's disease.

7.4.1 Baseline clinical characteristics were compared between patients and controls

Comparison between patients and controls of baseline clinical characteristics showed that all parameters were significantly different, except for LDLC, TC, ethnicity and gender. The patients were older, the median BMI was lower and they had a significantly worse atherogenic profile, as deduced by a preponderance of small dense LDL, higher TG, lower HDLC and higher hs-CRP levels (Table 42). As discussed in Chapter 5, a small sub-group was on lipid-lowering therapy, yet the adverse lipid profile persisted due to patients that were either not yet

initiated on lipid-lowering therapy or who had failed to achieve adequate lipid and lipoprotein targets on lipid-lowering therapy. On the other hand, patients had a lower BMI and lower NEFA levels, which may to some degree mitigate against the atherogenic potential from an adverse lipid profile. All the samples in both patients and healthy control subjects were taken in a non-fasted state, eliminating the postprandial state as a cause for the significant differences observed between these two groups. In view of these obvious differences, all future analyses were adjusted for BMI, age, gender and ethnicity for healthy control subjects, and in the case of patients, the analyses were adjusted for BMI, age, gender, ethnicity, foreign ancestry and hydrocortisone dose/m². For those parameters that were adjusted for, unadjusted comparisons are provided.

Table 42: Clinical characteristics in Addison's disease patients and controls

Clinical characteristics	**Patients *N*=147**	**Controls *N*=147**	***p*-value Patients versus controls**
Age in years (IQR)	46.0 (33.5-61.0)	41.5 (33.0-53.0)	**0.04***
Gender *N* (%) female male	 90 (61) 57 (39)	 90 (61) 57 (39)	1.00
Ethnicity White Mixed ancestry Asian Black	 97 (66) 34 (23) 5 (3) 11 (7)	 97 (66) 34 (23) 5 (3) 11 (7)	1.00
BMI kg/m² (IQR)	24.7 (22.1-30.3)	26.4 (24.1-31.2)	**0.007***
TG mmol/L (IQR)	1.67 (1.1-2.61)	1.39 (0.97-2.13)	**0.004***
TC mmol/L (SD)	5.70 (1.55)	5.77 (1.26)	0.64
HDLC mmol/L (IQR)	0.78 (0.53-1.07)	1.08 (0.93-1.27)	**<0.0001****
LDLC mmol/L (SD)	4.07 (1.37)	3.89 (1.17)	0.29
Proportion of small dense LDL *N* (%)	17/147 (12)	5/147 (3)	**0.01***
NEFA µmol/L (IQR)	341.0 (142.5-654)	467 (325.8-644.0)	**0.001***
Proportion on lipid lowering therapy *N* (%)	19/147 (13)	0 (0)	**<0.0001****

Clinical characteristics	Patients *N*=147	Controls *N*=147	*p*-value Patients versus controls
hs-CRP mg/L (IQR)	2.2 (0.97-6.38)	1.5 (0.64-3.25)	**0.01***

Median: Age, BMI, TG, HDLC and hs-CRP

Mean: TC and LDLC

BMI: Body mass index

TG: Triglyceride

TC: Total cholesterol

HDLC: High-density lipoprotein cholesterol

LDLC: Low-density lipoprotein cholesterol

LDL: Low-density lipoprotein

NEFA: Non-esterified fatty acids

IQR: Interquartile range

SD: Standard deviation

N: Number

p-value: Patients versus controls

$p < 0.05$ considered significant

$*p < 0.05$

$**p < 0.0001$

The observed genotype counts and frequencies, stratified by ethnicity and Addison's disease status, are shown in Table 43. While there were no differences observed for the ER22/23EK and N363S polymorphisms in patients and controls, there were significant differences in the distribution of the *Bcl*I genotypes (p = 0.02) among patients of different ethnic origins.

Table 43: Observed genotype counts and frequencies, stratified by ethnicity and Addison's disease status

			Patients		Controls	
Polymorphism	**Ethnicity**	**Genotype**	***N***	**freq**	***N***	**freq**
*Bcl*I						
C/C	White	Wild Type	34	0.38	43	0.45
G/C		Heterozygote	47	0.52	40	0.42
G/G		Homozygote	9	0.10	13	0.14
C/C	Mixed ancestry	Wild Type	18	0.53	22	0.65
G/C		Heterozygote	15	0.44	10	0.29
G/G		Homozygote	1	0.03	2	0.06
C/C	Asian	Wild Type	2	0.40	2	0.4
G/C		Heterozygote	2	0.40	2	0.4
G/G		Homozygote	1	0.20	1	0.2
C/C	Black	Wild Type	8	0.73	10	0.91
G/C		Heterozygote	3	0.27	0	0
G/G		Homozygote	0	0.00	1	0.09
p-value			**0.02***[a]		0.21[b]	
ER22/E23EK						
GAG AGG/ GAG AGG	White	Wild Type	84	0.93	88	0.92
GAA AAG/ GAG AGG		Heterozygote	6	0.07	8	0.08
GAG AGG/ GAG AGG	Mixed ancestry	Wild Type	33	0.97	33	0.97
GAA AAG/ GAG AGG		Heterozygote	1	0.03	1	0.03
GAG AGG/ GAG AGG	Asian	Wild Type	5	1.00	5	1
GAA AAG/ GAG AGG		Heterozygote	0	0.00	0	0
GAG AGG/ GAG AGG	Black	Wild Type	11	1.00	10	0.91
GAA AAG/ GAG AGG		Heterozygote	0	0.00	1	0.09
p-value			0.68[c]		0.60[d]	
N363S						
A/A	White	Wild Type	81	0.90	90	0.94
G/A		Heterozygote	9	0.10	6	0.06

			Patients		Controls	
A/A	Mixed ancestry	Wild Type	33	0.97	33	0.97
G/A		Heterozygote	1	0.03	1	0.03
A/A	Asian	Wild Type	5	1.00	5	1
G/A		Heterozygote	0	0.00	0	0
A/A	Black	Wild Type	11	1.00	11	1
G/A		Heterozygote	0	0.00	0	0
p-value			0.86[e]		0.87[f]	

Freq: Frequency

N: Number

$p < 0.05$ considered significant

$*p < 0.05$

A,C,G = DNA bases

rr: Comparison of the distribution of the *Bcl*I genotypes among patients of different ethnic origins

rs: Comparison of the distribution of the *Bcl*I 1 genotypes among controls of different ethnic origins

rt: Comparison of the distribution of the ER22/23EK genotypes among patients of different ethnic origins

ru: Comparison of the distribution of the ER22/23EK genotypes among controls of different ethnic origins

rv: Comparison of the distribution of the N363S genotypes among patients of different ethnic origins

rw: Comparison of the distribution of the N363S genotypes among controls of different ethnic origins

7.4.2 Race distribution among patients with BclI (C to G) polymorphism

A significant ethnicity-genotype association with *Bcl*I cases was observed, and thus all analyses were adjusted for race to control for possible population stratification (Figure 27).

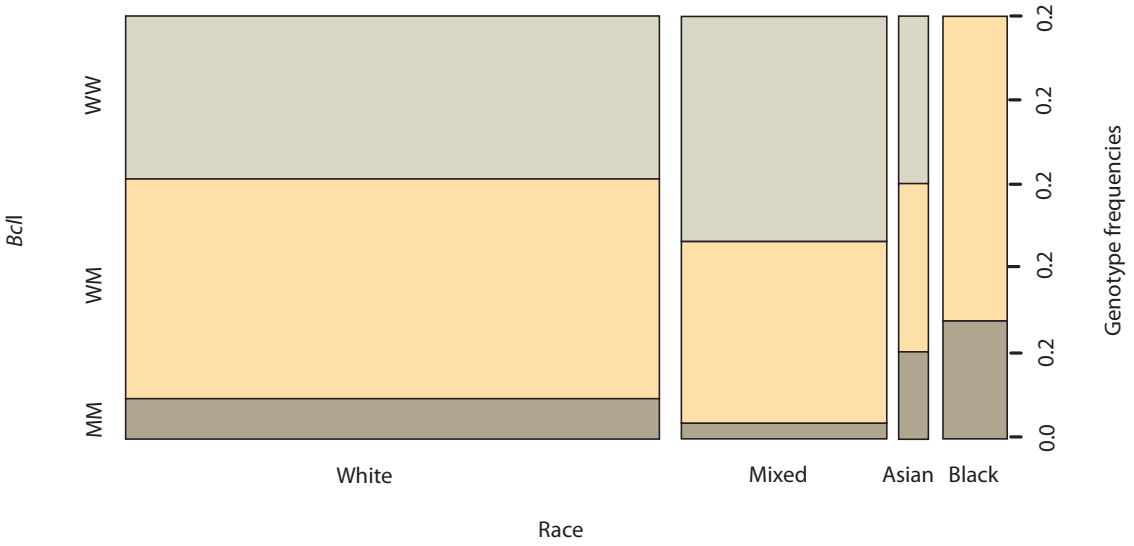


Figure 27: Mosaic plot of *BclI* genotype frequencies according to ethnicity in Addison's patients. The width of the blocks is proportional to the numbers of patients tested. Even taking into consideration the fact that white ethnicity represented the largest group tested, there was a still a greater frequency with which homozygous or heterozygous *BclI* polymorphism occurred.
MM: homozygous GG allele
WM: heterozygous GC allele
WW: wild type CC allele

The impact of gender, age and race (ethnicity) were explored in relation to the genotypes in both patients and controls, while the effect of foreign ancestry on the genotype of patients was examined (Table 44). Foreign ancestry was defined as any individual with a first- or second-degree relative from either Europe or USA. It is acknowledged that the vast majority of whites in South Africa have European ancestry, therefore it is expected that the genetic make-up of these subjects may mirror those of their European counterparts more closely compared to the other ethnic sub-groups. Gender and age did not influence the prevalence of any of the three polymorphisms examined in either patients or controls. On the other hand, race influenced the prevalence of the *BclI* polymorphism. Foreign ancestry did not influence the prevalence of any of the three polymorphisms.

Table 44: Gender, age, ethnicity and foreign ancestry associations with 3 glucocorticoid receptor polymorphisms (GCR), stratified by Addison's disease status

	Gender-genotype association[1] (*p*-value)		**Age-genotype association[2] (*p*-value)**		**Ethnicity-genotype association (*p*-value)**		**Foreign ancestry[3] (*p*-value)**
	Patients	Controls	Patients	Controls	Patients	Controls	Patients
*Bcl*I (C to G allele)	0.56	0.81	0.19	0.67	**0.02***	0.21	0.66
N363S (A to G allele)	0.51	0.43	0.25	0.51	0.86	0.60	0.50
ER22/23EK (GAG AGG to GAA AAG allele)	0.25	0.74	0.99	0.34	0.68	0.86	1

1: Comparisons made using Fisher exact test

2: Comparisons made using linear models

3: Foreign ancestry was only available for patients, thus no comparisons were made between patients and controls

A,C,G = DNA bases

$p < 0.05$ considered significant

*$p < 0.05$

7.4.3 Linkage disequilibrium

Linkage disequilibrium is when two alleles occur more commonly than is likely by random effects. It is known that linkage disequilibrium can differ between populations. Linkage disequilibrium (D') was calculated using a standard method in the programme R (obtainable from www.r-project.org).

There was significant linkage disequilibrium between *Bcl*I (G allele) and the N363S (G allele) polymorphisms in Addison's patients ($p = 0.03$), which was not

observed in controls (p = 0.93) (Table 45). On the other hand, there is a weak association for the G allele of the *Bcl*I and the GAA AAG allele of the ER22/23EK polymorphism in Addison's patients (p = 0.07). There was no linkage of the G allele of the N363S and the GAA AAG allele of the ER22/23EK in either the Addison's disease patients or their controls.

It is acknowledged that the results come from a small data set, comprising heterogeneous groups, and random effects may be seen. While significant linkage was identified with two alleles as shown above, the significance of this is uncertain and the results of this analysis should not be over-interpreted, as it is unlikely to confer meaningful significance.

Table 45: Linkage disequilibrium for three glucocorticoid receptor (GCR) polymorphisms in Addison's patients and controls

		N363S (A to G allele)		ER22/23EK (GAG AGG to GAA AAG allele).	
		Patients	**Controls**	**Patients**	**Controls**
*Bcl*I (C to G allele)	D	-0.01	-0.00	-0.00	0.00
*Bcl*I (C to G allele)	D'	1.0	0.05	1.0	0.01
*Bcl*I (C to G) allele	Corr.	-0.13	-0.00	-0.11	0.00
*Bcl*I (C to G allele)	Chi²	4.72	0.00	3.25	0.00
*Bcl*I (C to G allele)	p_1-value	**0.03***	0.93	0.07	0.95
	N	141$^{\beta}$	147	141$^{\beta}$	147
N363S (A to G allele)	D			-0.00	0.00
N363S (A to G allele)	D'			0.91	0.08
N363S (A to G allele)	Corr.			-0.02	0.07
N363S (A to G allele)	Chi²			0.22	1.37
N363S (A to G allele)	p_2-value			0.04	0.24
	N			141$^{\beta}$	147

D: Distance between two loci

D': The amount of linkage between two loci

p_1 *:* Comparison of the linkage disequilibrium observed between the *Bcl*I G allele and N363S G allele and ER22/23EK GAA AAG allele

p_2*:* Comparison of the linkage disequilibrium observed between the N363S G allele and the ER22/23EK GAA AAG allele

N: Number

Chi²: Chi-squared test

A,C,G = DNA bases

$p < 0.05$ considered significant

$*p < 0.05$

β: 6 patients with missing data

7.4.4 Comparisons of the prevalences of these polymorphisms between patients and controls

There were no gender, age or foreign ancestry related differences in the prevalences of the polymorphisms between patients and controls. The prevalence of the *Bcl*I (G allele) polymorphism analysed by race, differed between patients and controls (Table 45).

7.4.5 Frequencies of haplotype BclI-N363S-ER22/23EK

The frequencies of the various haplotype permutations are shown in Table 46. The combined wild type for each of the three polymorphisms was inferred in 62% of the patients and in 65% of the controls. The second most prevalent haplotype was *Bcl*I (G allele)/N363S (A allele)/ER22/23EK (GAG AGG allele), which occurred in 32% of patients and 29% of controls. All other haplotypes were observed at frequencies below 5%. The remaining possible permutations did not occur in this data set. There were no differences in the prevalences of the haplotypes between the patient and control groups.

Table 46: Frequencies of haplotype *Bcl*I-N363S-ER22/23EK

Polymorphism			Haplotype frequency		
*Bcl*I	N363S	ER22/23EK	Patients *N* (freq)	Controls *N* (freq)	*p*-value
W (C)	W (A)	W (GAG AGG)	91 (0.62)	96 (0.65)	0.53
M (G)	W (A)	W (GAG AGG)	47 (0.32)	43 (0.29)	0.51
W (C)	M (G)	W (GAG AGG)	6 (0.04)	3 (0.02)	0.50
W (C)	W (A)	M (GAA AAG)	3 (0.02)	4 (0.03)	0.72

N: Number

Freq: Frequency

M: Mutant

W: Wild type

A, C, G = DNA bases

p-value: Comparison of haplotype frequencies in patients and healthy control subjects

$p < 0.05$ considered significant

7.4.6 Clinical characteristics of the BclI (G allele) polymorphism in healthy control subjects and patients

Clinical characteristics were explored in healthy controls and patients with Addison's disease in relation to the *Bcl*I polymorphism (Table 47). The age and gender were no different among healthy controls of different *Bcl*I genotypes. In healthy controls, there were ethnic differences in the distribution of the *Bcl*I genotypes, as seen previously. For example, among white healthy controls, the most prevalent genotype was the wild type CC allele in 44%, while among healthy Asian and black controls, the heterozygous genotype (CG allele) was observed significantly less often than in their white and mixed ancestry counterparts. There were no ethnic differences in the distribution of the *Bcl*I genotypes among patients. However, when all the patients were compared to all the controls, there were differences in the distribution of the *Bcl*I genotypes. The clinical traits of BMI, gender, age and ethnicity were adjusted in both patients and controls. However, foreign ancestry and hydrocortisone/m^2 were adjusted only in patients. The TSH values were trimmed in the patient group because of an

extreme value of 31.2 mIU/L, in a single patient due to possible non-compliance with thyroxine replacement. Among healthy control subjects, there were no differences detected in TC, TG, HDLC, LDLC, NEFA, hs-CRP and the proportion with small dense LDL among those individuals who carried at least one G allele for the *Bcl*I polymorphism, compared to the wild type (two C alleles). In Addison's patients, there were no differences in the prevalence of hypertension, diabetes and use of lipid-lowering therapy, TC, TG, HDLC, LDLC, the proportion with small dense LDL, NEFA, hs-CRP, Framingham risk and AUC for salivary cortisol between those individuals who harbour at least one allele (G allele) for the *Bcl*I polymorphism and the wild type. Moreover, the total daily hydrocortisone dose did not differ between Addison's patients who harbour at least one G allele for the *Bcl*I polymorphism and the wild type (two C alleles). Comparison between Addison's patients and healthy controls, with respect to the *Bcl*I polymorphism, revealed that the TC, LDLC, small dense LDL, TG, HDLC, NEFA and hs-CRP did not differ when appropriately adjusted. On the other hand, there was a trend towards a greater BMI in the controls compared to the patients with respect to at least one G allele for the *Bcl*I polymorphism. In summary, the *Bcl*I (G allele) polymorphism had minimal effect on the clinical phenotype in both patients and healthy control subjects.

Table 47: Analysis of the effect of the *BclI* (C to G base change) in healthy control subjects and patients with Addison's disease

	Homozygous	Heterozygous	Wild type	p_1-value	p_2-value	p_3-value
controls *N* (freq) patients *N* (freq)	17 (0.12) 11 (0.08)	53 (0.36) 67 (0.48)	77 (0.52) 63 (0.44)			0.12
Age in years controls patients	 46.5 (26.0-54.0) 30.0 (20.5-50.5)	 41.0 (32.0-52.5) 45.0 (47.0-59.5)	 41.0 (34.0-53.0) 50.5 (34.0-63.8)	0.67[α]	0.19[β]	0.33[γ]
Gender controls females *N* (freq) patients females *N* (freq)	 11 (0.12) 6 (0.07)	 34 (0.38) 39 (0.44)	 45 (0.50) 43 (0.49)	0.77 [α]	0.54[β]	0.42[γ]
Ethnicity *N* (freq) White controls patients Mixed ancestry controls patients Asian controls patients Black controls patients	 13 (0.13) 9 (0.10) 2 (0.06) 1 (0.03) 1 (0.25) 1 (0.20) 1 (0.09) 0 (0)	 42 (0.43) 47 (0.52) 10 (0.29) 15 (0.44) 1 (0.25) 2 (0.40) 0 (0) 3 (0.27)	 43 (0.44) 35 (0.38) 22 (0.65) 18 (0.53) 2 (0.50) 2 (0.40) 10 (0.91) 8 (0.73)	**0.02***[α]	0.23[β]	**0.002***[γ]
BMI kg/m^2 (IQR) controls patients	29.4 (24.9-31.5) 22.5 (21.5-22.6)	26.0 (24.3-30.1) 25.0 (21.5-29.9)	26.5 (20.6-31.6) 27.4 (23.5-30.6)	0.23 [α]	0.11 [β]	0.05[γ]
Hypertension[Φ] *N* (freq)	0(0)	14 (0.64)	8 (0.36)	N/A	0.33	N/A
Diabetes[Φ] *N* (freq)	1 (0.05)	11 (0.55)	8 (0.40)	N/A	0.35	N/A
TG mmol/L (IQR) controls patients	 1.28(1.08-1.75) 1.96(1.28-2.56)	 1.31 (0.92-1.92) 1.82 (1.34-3.76)	 1.42 (0.98-2.15) 1.5 (1.05-2.0)	0.88	0.23	0.34
TC mmol/L (SD) controls patients	 5.92 (0.98) 5.94 (2.27)	 5.86 (1.27) 5.65 (1.65)	 5.67 (1.30) 5.74 (1.35)	0.72	0.79	0.85
HDLC mmol/L (IQR) controls patients	 1.15 (1.09-1.37) 0.68 (0.58-0.91)	 1.07 (0.91-1.37) 0.7 (0.54-0.98)	 1.06 (0.97-1.2) 0.86 (0.51-1.16)	0.77	0.71	0.50
LDLC mmol/L (SD) controls patients	 3.93 (1.0) 4.36 (1.26)	 3.82 (1.13) 4.02 (1.60)	 3.95 (1.21) 4.09 (1.07)	0.98	0.98	0.96
Proportion of small dense LDL *N* (freq) controls patients	 0 (0) 0 (0)	 2 (0.40) 12 (0.71)	 3 (0.60) 5 (0.29)	0.51	0.26	1.0
NEFA µmol/L (IQR) controls patients	 622.0 (388.5-939.8) 349.0 (196-448.0)	 444.0 (280-576) 226.0 (85.0-566.0)	 467.0 (327.0-642.0) 417.0 (179.0-707.0)	0.12	0.13	0.28
lipid lowering therapy[Φ] *N* (freq)	2 (0.11)	9 (0.47)	8 (0.42)	N/A	0.75	N/A
hs-CRP mg/L(IQR) controls patients	 2.2 (0.85-1.5) 0.54 (0.19-2.1)	 1.35 (0.60-2.28) 1.65 (0.97-3.0)	 1.55 (0.71-3.55) 1.9 (1.14-7.4)	0.34	0.74	0.36
Total daily hydrocortisone dose mg[Φ] (IQR)	25.0 (13.1-30.0)	23.8 (20.0-30.0)	20.0 (15.0-30.0)	N/A	0.742	N/A
Framingham risk (IQR) [Φ]	12.0 (5.3-22.0)	14.4 (8.0-27.4)	13.0 (4.3-21.9)	N/A	0.39	N/A
TSH mIU/L (IQR)[Φ]	1.23 (0.90-2.46)	1.21 (0.50-1.91)	1.66 (0.93-2.18)	N/A	0.2	N/A
AUC nmol*min*L^{-1} (IQR)[Φ]	431.0 (24.0-160.0)	271.0 (150.0-624.0)	760.0 (575.0-945.0)	N/A	0.24	N/A

Median: Age BMI, TG, HDLC, NEFA, hs-CRP, total daily hydrocortisone dose, TSH and AUC

Mean: TC and LDLC

BMI Body mass index
TG: Triglyceride
TC: Total cholesterol
HDLC: High-density lipoprotein cholesterol
LDLC: Low-density lipoprotein cholesterol
LDL: Low-density lipoprotein
NEFA: Non-esterified fatty acids
hs-CRP: Highly sensitive C-reactive protein
mg: Milligrams
TSH: Thyroid stimulating hormone
AUC: Area under the curve (salivary cortisol)
Φ: parameter examined in patients only
‡: All female subjects
Freq: Frequency
N: Number
SD: Standard deviation
IQR: Interquartile range

α: Unadjusted *p*-values for controls
β: Unadjusted *p*-values for patients
γ: Unadjusted *p*-Values for comparison of patients with controls
N/A: Not applicable

p-values for total number, age, gender and ethnicity were unadjusted
p_1-value: Comparing *Bcl*I genotypes among control subjects, adjusted for BMI, age, gender and ethnicity
p_2-value: Comparing *Bcl*I genotypes among Addison's patients, adjusted for BM), age, gender, ethnicity, foreign ancestry and hydrocortisone dose per metre squared
p_3-value: Simultaneous/joint comparison between *Bcl*I genotypes, and between Addison's patients and healthy control subjects (interaction), adjusted for BMI, age, gender and ethnicity
$p < 0.05$ considered significant
$^*p < 0.05$

7.4.7 Clinical characteristics of the ER22/23EK (GAA AAG base changes) polymorphisms in healthy controls and in patients with Addison's disease

Clinical characteristics were explored in both the healthy control subjects and patients with Addison's disease in relation to the ER22/23EK polymorphism (Table 48). There were no ethnic differences in the distribution of the ER22/23EK (GAA AAG base changes) polymorphism in patients and controls, and between patients and controls. There was no difference in age and gender among healthy controls, among Addison's patients, and between healthy control subjects and patients of different ER22/23EK genotypes. The clinical traits of BMI, gender, age and ethnicity were adjusted in both patients and controls. However, foreign ancestry and hydrocortisone/m² were adjusted only in the patients.

In both the healthy control group and the Addison's disease patient group, the BMI was considerably greater in the heterozygote subjects compared to the wild types and there was no difference when the patients were compared to all the healthy controls in relation to the ER22/23EK polymorphism. The LDL was lower in the heterozygote sub-group compared to the wild type group of healthy control subjects, but this relationship was not observed in Addison's patients. Moreover, there was no difference in LDL when all the patients were compared to all the healthy controls.

The TC, TG HDLC, NEFA and the proportion of small dense LDL were not affected by the presence of the ER22/23EK polymorphism among healthy control subjects when adjusted for BMI, age, gender, ethnicity and foreign ancestry. Similarly, the TC, TG HDLC, NEFA, proportion of small dense LDL and hs-CRP were not affected by the presence of the ER22/23EK polymorphism among Addison's patients when appropriately adjusted. One patient was excluded from the analysis because of a very high TSH due to non-compliance with thyroxine replacement for primary hypothyroidism. The prevalence of hypertension, diabetes and the use of lipid-lowering therapy among Addison's patients were not influenced by the presence

of this polymorphism when adjusted. Within the context of this polymorphism, the HDLC was lower in all the patients compared to all the controls. However, the proportion with small dense LDL, TC, LDLC, TG, NEFA, Framingham risk and hs-CRP did not differ between patients and controls in relation to this polymorphism. In summary, the BMI was higher in the heterozygote versus wild types in both patients and controls. The LDLC was lower among heterozygote controls compared to the wild type healthy control subjects, and the HDLC was lower in patients compared to healthy control subjects examined in relation to the presence of the ER22/23EK polymorphism. In summary, the GAA AAG base changes resulting in the ER22/23EK polymorphism was associated with an increased BMI in both controls and patients compared to the wild type. This polymorphism was also associated with a reduction in LDLC in healthy controls and HDLC was found to be lower when comparing all the patients to all the healthy control subjects.

Table 48: Analysis of the effect of the presence of the ER22/23EK (GAG AGG to GAA AAG base changes) polymorphism in healthy controls and patients with Addison's disease

	Heterozygotes	Wild type	p_1-value	p_2-value	p_3-value
controls *N* (freq) patients *N* (freq)	10 (0.07) 7 (0.05)	136 (0.93) 134 (0.95)			0.62
Age in years (IQR) controls patients	 40.5 (29.0-47.0) 27.0 (22.5-54)	 42.0 (33.0-53.0) 43.0 (35.0-62.0)	0.35$^{\alpha}$	0.99$^{\beta}$	0.06$^{\gamma}$
‡Gender *N* (freq) controls females *N* (freq) patients females *N* (freq)	 7 (0.08) 6 (0.07)	 83 (0.92) 82 (0.93)	0.74$^{\alpha}$	0.25$^{\beta}$	0.45$^{\gamma}$
Ethnicity *N* (freq) Whites controls patients Mixed Ancestry controls patients Asians controls patients Blacks controls patients	 8 (0.08) 6 (0.07) 1 (0.03) 1 (0.03) 0 (0) 0 (0) 1 (0.09) 0 (0)	 88 (0.92) 85 (0.93) 33 (0.97) 33 (0.97) 5 (1.0) 5 (1.0) 10 (0.91) 11 (1.0)	0.68$^{\alpha}$	0.86$^{\beta}$	0.88$^{\gamma}$
BMI kg/m^2 (IQR) controls patients	26.3 (23.9-30.6) 29.4 (28.1-35.4)	24.2 (27.8-38.0) 24.7 (22.1-30.0)	**<0.0001**$^{**\alpha}$	**0.02**$^{*\beta}$	0.75$^{\gamma}$
Hypertension$^{\Phi}$ N (freq)	0 (0)	22 (1.0)	N/A	0.21	N/A
Diabetes$^{\Phi}$ *N* (freq)	0 (0)	20 (1.0)	N/A	0.98	N/A
TG mmol/L (IQR) controls patients	 1.7 (1.41-1.92) 2.18 (1.55-2.76)	 1.34 (0.95-2.14) 1.65 (1.1-2.5)			
TC mmol/L (SD) controls patients	 5.48 (0.53) 5.27 (0.97)	 5.79 (1.30) 5.77 (1.54)	0.09	0.22	0.60
HDLC mmol/L (IQR) controls patients	 1.11 (1.06-127) 0.72 (0.53-0.86)	 1.07 (0.92-1.27) 0.78 (0.54-1.09)	0.60	0.12	**0.04***
LDLC mmol/L (SD) controls patients	 3.46 (0.51) 3.52 (0.78)	 3.93 (1.20) 4.10 (1.39)	**0.02***	0.41	0.97
Proportion of small dense LDL *N* (freq) controls patients	 0 (0) 0 (0)	 5 (1.0) 17 (1.0)	0.11	0.41	0.26
NEFA µmol/L (IQR) controls patients	 550.5 (365.0-681.3) 450.0 (192.0-658.0)	 457.0 (327.0-642.0) 329.0 (139.0-654.0)	0.66	0.65	1.0
lipid lowering therapy$^{\Phi}$ *N* (freq)	0 (0)	19 (1.0)	N/A	0.981	N/A
hs-CRP mg/L (IQR) controls patients	 4.2 (1.1-7.2) 5.0 (3.4-8.05)	 1.5 (0.62-2.85) 2.2 (1.03-6.4)	0.96	0.74	0.69
Total daily hydrocortisone dose mg$^{\Phi}$ (IQR)	30.0 (25.0-30.0)	20.0 (20.0-30.0)	N/A	0.32	N/A
Framingham risk (IQR) $^{\Phi}$	9.0 (3.1-16.7)	13.3 (5.9-25.7)	N/A	0.94	N/A
TSH$^{\Phi}$ mIU/L (IQR)	1.10 (0.56-1.38)	1.46 (0.78-2.16)	N/A	0.88	N/A
AUC nmol*min*L^{-1} (IQR) $^{\Phi}$	166.0 (140.0-188.0)	404 (188-1126.0)	N/A	0.31	N/A

Median: Age, BMI, TG, HDLC, NEFA, hs-CRP, total daily hydrocortisone dose, TSH and AUC

Mean: TC and LDLC

BMI: Body mass index

TG: Triglyceride

TC: Total cholesterol

HDLC: High-density lipoprotein cholesterol

LDLC: Low-density lipoprotein cholesterol

LDL: Low-density lipoprotein

NEFA: Non-esterified fatty acids

hs-CRP: Highly sensitive C-reactive protein

mg: Milligrams

TSH: Thyroid stimulating hormone

AUC: Area under the curve (salivary cortisol)

Φ: Parameter examined in patients only

‡: All female subjects

Freq: Frequency

N: Number

SD: Standard deviation

IQR: Interquartile range

α: Unadjusted *p*-values for controls

β: Unadjusted *p*-values for patients

γ: Unadjusted *p*-values for comparison of patients with controls

N/A: Not applicable

p-values for total number, age, gender and ethnicity were unadjusted

p_1-value: Comparing ER22/23EK genotypes among control subjects adjusted for BMI, age, gender and ethnicity

p_2-value: Comparing ER22/23EK genotypes among Addison's patients adjusted for BMI, age, gender, ethnicity, foreign ancestry and hydrocortisone dose per metre squared

p_3-value: Simultaneous/joint comparison between ER22/23EK genotypes, and between Addison's patients and healthy control subjects (interaction) adjusted for BMI, age, gender and ethnicity

$p < 0.05$

$^{*}p < 0.05$

$^{**}p < 0.0001$

7.4.8 Clinical characteristics of the N363S (A to G base change) polymorphism in healthy control subjects and patients with Addison's disease

Clinical characteristics were explored in the healthy control subjects and in patients with Addison's disease in relation to the N363S polymorphism (Table 49). The distribution of N363S polymorphism did not differ between the patients and controls. The unadjusted age and gender were no different among healthy controls, among Addison's patients, and between healthy control subjects and patients of different N363S genotypes. Ethnicity did not influence the distribution of the N363S genotype in either healthy control subjects or Addison's patients. Moreover, when all the patients were compared to all the controls, no differences in ethnic distribution of the N363S polymorphism were discerned. The clinical traits of BMI, gender, age and ethnicity were adjusted in both patients and controls. However, foreign ancestry and hydrocortisone/m^2 were only adjusted in patients. As before, TSH values were trimmed in the patients because of an extreme value of 31.2 mIU/ml from a single subject, likely due to non-compliance of thyroxine replacement for primary hypothyroidism. Among healthy control subjects, there were no differences in TC, TG, HDLC, the proportion with small dense LDL, NEFA and hs-CRP among those individuals who carried at least one G allele for the N363S polymorphism, compared to the wild type (two A alleles) when appropriately adjusted. In Addison's patients, there were no differences in the prevalence of hypertension, diabetes and the use of lipid-lowering therapy, TC, TG, HDLC, LDLC, proportion with small dense LDL, NEFA, hs-CRP, Framingham risk and AUC for salivary cortisol between those individuals who harbour at least one allele (G allele) for the N363S polymorphism and the wild type with appropriate adjustment. Moreover, hydrocortisone dose/m^2 did not differ between Addison's patients who harbour at least one G allele for the N363S polymorphism and the wild type (two A alleles). Also, when all the patients were compared with all the healthy controls, the TC, LDLC, small dense LDL, TG, HDLC, NEFA and hs-CRP, when appropriately adjusted, did not differ with respect to the N363S polymorphism. In summary, the N363S polymorphism did not appear to influence

any of the clinical characteristics that were examined in this study.

Table 49: Analysis of the effect on the presence of N363S (A to G base change) polymorphisms in healthy controls and patients with Addison's disease

		Heterozygotes	Wild type	p_1-value	p_2-value	p_3-value
Controls *N* (freq) patients *N* (freq)		7 (0.05) 10 (0.07)	140 (0.95) 131 (0.93)			0.460
Age in years				0.51[α]	0.25[β]	0.2[γ]
	controls	49.0 (40.5-49.3)	41.0 (33.0-53.0)			
	patients	31.5 (23.3-44.8)	41.0 (34.3-60.8)			
‡Gender				0.43[α]	0.50[β]	0.08[γ]
controls females *N* (freq)		3 (0.03)	87 (0.97)			
patients females *N* (freq)		5 (0.06)	82 (0.94)			
Ethnicity *N* (freq)				0.86[α]	0.60[β]	0.68[γ]
White	controls	6 (0.06)	90 (0.94)			
	patients	9 (0.1)	81 (0.9)			
Mixed ancestry	controls	1 (0.03)	33 (0.97)			
	patients	1 (0.03)	33 (0.97)			
Asian	controls	0 (0)	5 (1.0)			
	patients	0 (0)	5 (1.0)			
Black	controls	0 (0)	11 (1.0)			
	patients	0 (0)	11 (1.0)			
BMI kg/m^2 (IQR)	controls	29.9 (26.5-31.0)	24.8 (22.1-30.4)	0.18[α]	0.73[β]	0.30[γ]
	patients	25.9 (22.9-27.2)	24.8 (22.1-30.4)			
Hypertension *N* (freq) [Φ]		2 (0.09)	20 (0.91)	N/A	0.07	N/A
Diabetes frequency *N* (freq) [Φ]		0 (0)	20 (1.0)	N/A	0.084	N/A
TG mmol/L (IQR)	controls	1.7 (1.47-1.79)	1.35 (0.93-2.13)	0.44	0.70	0.29
	patients	1.52 (1.16-2.06)	1.71 (1.1-2.62)			
TC mmol/L (SD)	controls	6.14 (1.15)	5.75 (1.27)	0.65	0.96	0.71
	patients	5.65 (1.64)	5.73 (1.54)			
HDLC mmol/L (IQR)	controls	1.03 (1.03-1.11)	1.08 (0.93-1.27)	0.83	0.52	0.29
	patients	0.88 (0.69-0.93)	0.77 (0.51-1.08)			
LDLC mmol/L (SD)	controls	3.8 (0.32)	3.90 (1.20)	0.70	0.64	0.83
	patients	4.12 (1.48)	4.08 (1.38)			
Proportion of small dense LDL *N* (freq)				0.63	0.36	0.26
	controls	0 (0)	5 (1.0)			
	patients	0 (0)	17 (1.0)			
NEFA µmol/L (IQR)				1.0	0.40	0.26
	controls	351.0 (350.0-672.0)	467.0 (327.0-642.0)			
	patients	299.0 (205.0-486.0)	341.0 (131.0-692.0)			
Proportion on lipid lowering therapy *N* (freq) [Φ]		2 (0.11)	17 (0.89)	N/A	0.79	N/A
hs-CRP mg/L (IQR)	controls	2.4 (1.95-3.9)	1.5 (0.62-2.9)	0.44	0.15	0.32
	patients	1.12 (0.53-2.73)	2.5 (1.5-6.9)			
Total daily hydrocortisone dose mg (IQR) [Φ]		25.0 (20.0-30.0)	20.0 (20.0-30.0)	N/A	0.20	N/A
Framingham risk (IQR) [Φ]		8.7 (7.4-26.2)	13.7 (3.5-22.3)	N/A	0.31	N/A
TSH mIU/L (IQR) [Φ]		1.63 (0.94-1.84)	1.38 (0.76-2.15)	N/A	0.18	N/A
AUC nmol*min*L^{-1} (IQR) [Φ]		131.0 (118.0-143.0)	404.0 (203.0-126.0)	N/A	0.20	N/A

Median: Age BMI, TG, HDLC, NEFA, hs-CRP, total daily hydrocortisone dose, TSH and AUC

Mean: TC and LDLC
BMI: Body mass index
TG: Triglyceride
TC: Total cholesterol
HDLC: High-density lipoprotein cholesterol
LDLC: Low-density lipoprotein cholesterol
LDL: Low-density lipoprotein
NEFA: Non-esterified fatty acids
hs-CRP: Highly sensitive C-reactive protein
mg: Milligrams
TSH: Thyroid stimulating hormone
AUC: Area under the curve (salivary cortisol)
Φ: Parameter examined in patients only
‡: All female subjects
Freq: Frequency
N: Number
SD: Standard deviation
IQR: Interquartile range

α: Unadjusted *p*-values for controls
β: Unadjusted *p*-values for patients
γ: Unadjusted *p*-values for comparison of patients with controls
N/A: Not applicable

p-values for total number, age, gender and ethnicity were unadjusted
p_1-value: Comparing N363S genotypes among control subjects adjusted for BMI, age, gender and ethnicity
p_2-value: Comparing N363S genotypes among Addison's patients adjusted for BMI, age, gender, ethnicity, foreign ancestry and hydrocortisone dose per metre squared
p_3-value: Simultaneous/joint comparison between N363S genotypes, and between Addison's patients and healthy control subjects (interaction) adjusted for BMI, age, gender and ethnicity
$p < 0.05$ considered significant
*$p < 0.05$

7.5 Discussion

This is the first study to examine the effect of the common GCR polymorphisms in patients with hypoadrenalism and to correlate polymorphisms with either dose of hydrocortisone or with metabolic parameters.

The principal metabolic alteration conferred by the GCR polymorphisms was an elevated unadjusted BMI in healthy controls and patients, in the presence of the ER22/23EK polymorphism. However, the LDLC was reduced in heterozygous healthy control subjects when adjusted for BMI, foreign ancestry, gender and ethnicity. The ER22/23EK polymorphism was associated with a lower HDLC in patients compared to controls. Neither the *Bcl*I nor the N363S polymorphisms were associated with any significant alteration in any of the metabolic clinical traits examined in this study.

Since the prevalence of these GCR polymorphisms was similar among patients and healthy control subjects, no role for this gene in the pathogenesis of Addison's disease in this South African Addison's disease cohort was inferred. However, there were significant differences in the distribution of the *Bcl*I genotypes among patients of different ethnic origins, with a greater proportion of white ancestry subjects harbouring the G allele compared to the other ethnic groups. The small sample size could have influenced the distribution of this polymorphism among white ancestry subjects. The observed G allele frequency for *Bcl*I polymorphism in the NCBI database was 67%,[7] whereas in the Addison's patients and controls, the G allele frequency was 60% and 53% respectively. The reduced frequency in Asian and black races, in both the patients and controls, may account for the disparity observed between the NCBI database and the Addison's patients and their respective controls. In addition, the small sample sizes could have accounted for the observed low frequency. The observed GAA AAG allele frequency for the ER22/23EK polymorphism in the NCBI database varied between 0.02% and 3.4%, whereas in the Addison's patients and controls, the GAA AAG allele

was 5% and 7% respectively, which is substantially greater than the available data from the NCBI database. The G allele frequency observed for the N363S polymorphism in the NCBI database varied between 0% and 8.3%, whereas in the Addison's patients and controls, the observed G allele frequency was 5% and 7% respectively. In keeping with the NCBI database, the greatest frequency of the G allele was observed among the white patients. It is likely that the heterogeneous ethnic make-up accounts for the differences observed in this South African cohort study compared to the NCBI database.[7] Further population-based studies are warranted to confirm these findings.

The ER22/23EK polymorphism appeared to have the greatest impact in its association with an elevated BMI among both healthy control subjects and the Addison's disease patients. The effect and magnitude of the ER22/23EK polymorphism on elevating the BMI in the study of Addison's disease is intriguing and has not been reported previously. If this is a true association, it would have been revealed in other studies of obesity, especially since a difference of 2 kg/m^2 in BMI was observed in this study. Similarly, a genome-wide scan for obesity showed that single nucleotide polymorphisms (SNPs), located on chromosome 16, were most likely to affect BMI, hip circumference and mass.[8] While a genome-wide scan assessing susceptibility for T2DM in obese and non-obese subjects demonstrated four loci, none of them was related to chromosome 5, indicating that the SNP ER22/23EK was not associated with obesity in previous studies.[9] As far as could be determined, previous genome-wide association scans failed to demonstrate an association between obesity and any of the GCR polymorphisms. It was expected that this polymorphism should have resulted in a reduced in either a reduced or unchanged BMI. Therefore, the implication is that the presence of this polymorphism does not confer either an obligatory metabolically protective or reduced GC sensitivity phenotype. This finding of an elevated BMI, and in particular the magnitude of its effect size, in association with this polymorphism, is thus counterintuitive, and as it is the first study of its kind to show a positive

association, substantiation of this unforeseen association is warranted in future studies.

Additionally, the ER22/23EK (GAA AAG allele) polymorphism was associated with a reduction in LDLC in healthy control subjects but not in Addison's patients. The absolute LDLC reduction was 0.47 mmol/L which was less than the 0.80 mmol/L observed by van Rossum et al.[10] The explanation for the greater reduction in LDLC may lie in the age difference (average age of 40.5 years for the healthy control subjects versus 69.2 years in the study by Rossum et al) and discrepant mean LDL levels (3.46 mmol/L in the healthy control subjects versus 4.31 mmol/L in the elderly Dutch population study).[10] There is evidence to support an increasing LDL with age, and it is conceivable that a higher baseline LDL may be modified to a greater degree by the ER22/23K polymorphism.[11]

It remains a challenge to reconcile the differential effect of this polymorphism on healthy control subjects, in which a reduction in LDL was observed compared to Addison's patients, who demonstrated no change in LDL. As there were few subjects who were heterozygous for the ER22/23EK polymorphism, the possibility of a lack of statistical power needs to be entertained. Addison's patients who were heterozygous for this polymorphism also exhibited lower LDL levels than the wild type, but this did not reach statistical significance. Patient LDL levels were scattered more widely (higher standard deviations), possibly impairing the ability to detect a statistical significance.

Pharmacological or supra-physiological doses of GCs could cause an increase in LDL,[12-14] and the beneficial effect of the ER22/23EK polymorphism may be negated by the possibility of supra-physiological hydrocortisone replacement. On the other hand, no correlation was found in this study between hydrocortisone dose and LDL (as seen in Chapter 5), invoking the possibility that the reduction in LDL in association with this polymorphism in healthy controls, but not in

Addison's patients, is through an entirely different mechanism. In this cohort of both patients and controls, there was no evidence for coexistent competing GCR polymorphisms in the same patient, that is, sensitising and resistant haplotypes, ruling this out as a potential mechanism for selective LDL reduction in the healthy control subjects. Some studies have confirmed a favourable metabolic profile in relation to the ER22/23EK polymorphism. This beneficial effect on LDLC was not always universal, and one study confers benefit by trend and fails to reach statistical significance.[10 15-17] There are no clear explanations for the difference in LDLC response between Addison's patients and controls. Overall, these polymorphisms were very poorly predictive for a specific clinical or metabolic phenotype.

The ER22/23EK (GAA AAG allele) polymorphism was also associated with a decreased HDLC in patients compared to healthy controls. However, this observation was seen neither in the healthy controls nor the Addison's patients as independent groups. Already, prior to examining the GCR polymorphisms, the Addison's patients exhibited a significantly lower HDLC compared to their healthy controls, which suggests that this discrepant relationship may be exaggerated when examining the polymorphisms. As the ER22/23EK polymorphism is associated with decreased GC sensitivity, it is speculated that it in turn may result in lower HDLC levels[18] due to possible coexistent inflammation. However, the ER22/23EK polymorphism is associated with decreased GC sensitivity and a favourable CV lipid profile, and thus the lower HDLC is counterintuitive.[19] It is not clear whether this polymorphism may have a greater influence in Addison's patients, as they may have a greater coexistent acute phase response compared to healthy control subjects, which accounts for the marked differences in HDL.[18] The study by van Rossum et al did not show a difference in HDL in relation to the ER22/23EK polymorphism, and as far as is known, there are no previous studies to support the finding of a low HDL associated with this polymorphism.[17] The possibility that this represents a chance finding cannot be excluded.

The *Bcl*I and the N363S polymorphisms did not appear to alter the clinical traits significantly. The small sample of patients may have contributed to the lack of association. As these polymorphisms only modestly alter the phenotype, it is not surprising that this study failed to show an association with the *Bcl*I and the N363S polymorphism. There are several negative studies examining the influence of these three polymorphisms in different clinical scenarios. Donn et al showed no association between the ER22/23EK polymorphism and rheumatoid arthritis.[20] Decorti et al and De Iudicibus et al similarly found no association with inflammatory bowel disease and both the ER22/23EK and N363S polymorphisms respectively.[21 22] Spijker et al failed to find association with the latter polymorphism and bipolar affective disorder.[23] Lee et al identified no association between the *Bcl*I polymorphism and rheumatoid arthritis in Korean subjects.[24]

Harbouring a polymorphism does not invariably give rise to a clinically significant alteration or abnormal phenotype. There are a number of negative studies that show no metabolic derangements in association with polymorphisms that are expected to enhance GC sensitivity. In a study by van Rossum et al, elderly Dutch individuals who demonstrated increased sensitivity to dexamethasone, counterintuitively had lower BMI and a tendency towards lower lean mass, whereas fat mass was no different in individuals harbouring the *Bcl*I polymorphism compared to the wild type genotype.[25] The incongruity of these studies may corroborate the tenuous effect that these polymorphisms confer on modifying the clinical phenotype.

A strong linkage disequilibrium exists between *Bcl*I (G allele) and N363S (G allele) in the South African Addison's patients. This has not been observed previously. As the combination of these alleles could contribute to enhance glucocorticoid sensitivity, it is difficult to reconcile how the combination of these alleles may predispose to either the development or the progression of Addison's disease. It is not likely that any of the GCR polymorphisms could confer an increased

risk for the development of Addison's disease. Since this data comes from a small group, which was heterogeneous and random effects may be seen, it is unlikely to be of any meaningful clinical significance. On the other hand, researchers have examined the relationship between ACTH-secreting tumours and GCR polymorphisms. The N363S polymorphism occurred in 17% of ACTH-secreting tumours in one study, but this was unlikely to be pathogenic as the latter polymorphism resulted in increased sensitivity of cortisol, and a polymorphism inducing resistance would more likely explain the molecular defect, which was not detected.[26]

There are isolated studies that have explored the relationships between steroid dose and GCR polymorphisms. Szczepankiewicz et al showed that there was no association between any of the GCR polymorphisms and the need for increased doses of GCs for asthma.[27] Similarly, in this cohort of South African Addison's disease patients, no association was found between hydrocortisone dose and the various GCR polymorphisms, even when adjusting for hydrocortisone/m^2. In addition, the AUC for salivary cortisol was not associated with any of the GCR polymorphisms examined. Intracellular cortisol accumulation, as measured by ^{3}H-cortisol, is substantially lower than the amount with which neuronal tissue was incubated, but the major intracellular cortisol is unbound to the GCR.[28] Therefore, as the amounts of cortisol that bind to the receptors are very small compared to the levels in the circulation, it was considered unlikely therefore that the AUC for salivary cortisol would be affected by the GCR polymorphisms.[29] Further, the main clearance of plasma cortisol is via hepatic (production of glucuronides) and renal metabolism, where the 11 beta-hydroxysteroid-dehydrogenase-II converts cortisol to cortisone, in addition to the production of 17-hydroxycorticoids.[30]

Very few studies have examined the association between AUC for cortisol exposure and GCR polymorphisms. Similarly, no previous studies, to my knowledge, have examined AUC for salivary cortisol and correlated this with GCR polymorphisms

in primary hypoadrenalism. In two of these three alleles which occur at low frequencies, the possibility of false negative results should be considered.

There are a number of potential pitfalls in performing association studies with polymorphisms and phenotyping. Accuracy can be enhanced by increasing the sample size and thereby minimising inter-research variation. Indeed, the possibility of performing a type I error can occur with multiple testing. Additionally, it has been suggested that these polymorphisms can exert different effects in different ethnic groups, and ideally, a homogenous population should be selected in order to determine the true effect.[31]

It was assumed that patients harbouring a sensitising GCR polymorphism may require lower doses of hydrocortisone, whereas individuals with a polymorphism inducing a degree of GC resistance may require higher doses of hydrocortisone, by virtue of persistent symptoms of GC insufficiency.[32] Nevertheless, no relationship between GCR polymorphisms and hydrocortisone doses was found in this study. This may possibly be explained by the fact that hydrocortisone doses were prescribed on a totally empiric basis by their treating doctor.

7.5.1 Weaknesses of this study

The small number of participants with this rare condition may have contributed to the lack of associations found. The 9β polymorphism of the GCR gene has been associated with an increased risk of myocardial infarction, especially in an elderly sub-group. Those who were homozygous for this polymorphism had increased intima media thickness and an almost threefold increased risk of CVD,[33] therefore analysis of this polymorphism may have yielded interesting data in the South African Addison's disease patients.

7.5.2 Conclusion

The effect of the GCR polymorphisms in this cohort was to increase the BMI

in healthy controls and patients harbouring the ER22/23EK polymorphism. This was in contrast to what was expected, considering that it normally reduces cortisol sensitivity. Additionally, no relationship was found between the doses of hydrocortisone and the presence of any of the *Bcl*I, N363S and ER22/23EK GCR polymorphisms. Further work is required to confirm whether these findings can be corroborated in a larger series of Addison's patients on replacement doses of GCs.

7.6 References

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Chapter 8
Final discussion, recommendations and concluding remarks

8.1 Summary of the major findings

Utilising a systematic classification of Addison's disease, it was possible to confirm that autoimmune Addison's disease occurred in more than 50% of this cohort, contradicting an earlier study suggesting that autoimmune Addison's disease is uncommon in South Africa.[1 2] In keeping with several research groups, HLA DQB1*0201 and *0302 alleles were associated with APS2 when patients with T1DM were excluded.[3-6] Despite the burden of the two epidemics that South Africa is currently facing, tuberculosis as a cause for Addison's disease[7 8] was remarkably uncommon and none of the Addison's disease in this cohort was caused by either HIV or AIDS.[9 10] Although there were few black or Asian South Africans in the cohort, none had adrenal autoantibodies. Further as found by Tanaka et al, ACA were less detectable than 21-hydroxylase autoantibodies, long after the onset of Addison's disease.[11]

Several lipid and lipoprotein parameters were worse in patients compared to controls, indicating that Addison's disease patients may have a predisposition to abnormal lipids and lipoproteins. Approximately 65% of patients exhibited hypercholesterolaemia of >5.0 mmol/L, hypertriglyceridaemia >1.7 mmol/L was identified in approximately 50%, about 75% of patients had an HDLC <1.0 mmol/L and 75% demonstrated an LDLC >3.0 mmol/L. Coexistent diabetes mellitus increased moderate levels of hypertriglyceridaemia and further raised severely elevated LDL levels. South African Addison's patients demonstrated a more adverse lipid profile than the matched Swedish patients. An increased incidence of small dense LDL and elevated hs-CRP concentrations was identified. This comprehensive analysis has established a probable causal link between Addison's disease and premature CV mortality,[12] which contributes significantly

to the body of knowledge in this area.

Based on the salivary cortisol data, Addison's disease patients had a much greater cortisol exposure than that generated by the healthy control subjects' endogenous cortisol production. The median dose of hydrocortisone was 12 mg/m^2 and as such is likely to represent supra-physiological concentrations given that the normal daily cortisol production is 6-11 mg/m^2.[13] The peak salivary cortisol correlated well with the AUC in both patients and healthy control subjects. Thus the peak concentration may be used as a surrogate marker for monitoring AUC in both Addison's patients and healthy control subjects, limiting the need for multiple sampling and reducing the attendant expense.

The predominant metabolic changes associated with the ER22/23EK GCR polymorphism were an elevated BMI in Addison's disease patients and healthy control subjects. These findings were entirely counterintuitive as this polymorphism ostensibly confers a degree of GC resistance.[14] A lower LDLC was associated with the ER22/23EK polymorphism in healthy control subjects only, and may be partially explicable by GCR resistance. Neither the *Bcl*I nor the N363S polymorphisms were associated with alterations in any of the studied metabolic clinical characteristics.

8.2 Strengths of the study

The relatively large size of this cohort is a strength of the study. The cohort was derived from several sources, including databases from medical insurance companies, a database generated by the researcher of private and public health-care and a commercial database. It sought to enrol patients from all geographical regions throughout a large country to minimise selection bias by geographical area.

Sera were subjected to a large panel of appropriate autoantibodies and DNA was collected for genetic studies. At least two confirmatory adrenal autoantibodies were screened for, as it is known that 21-hydroxylase autoantibodies are more sensitive than the immunofluorescence assay of ACA in cases with overt primary adrenal failure.[11] Addison's patients were compared to healthy control subjects and several cohort studies of lipids and lipoproteins in the community,[15-18] providing at least two strata for comparison with healthy subjects. Unlike the study by Giordano et al, the South African Addison's disease study elucidated in detail the impact of the combination of Addison's disease and diabetes on lipids and lipoproteins.[19] Three biochemical markers of CVD were also examined in this study including small dense LDL, hs-CRP and NEFA; two of these have established roles in evaluating patients for CVD risk.[20 21]

The salivary cortisol was measured 16 times in a 16 hour period in this study, which is considerably longer than many others in an exclusively primary adrenal cohort.[22-27] It also showed that it is possible to monitor replacement therapy in rural and isolated regions of a vast country, where infrastructure is poor.

The study of GCR polymorphisms in Addison's disease is novel and provides an insight, as to how this polymorphism could be altering the clinical phenotype in this condition.

8.3 Weaknesses of the study

The study has a number of weaknesses. Its cross-sectional design ensured that several cases were included many years after the diagnosis was made. There were missing biochemical data, confirmatory of Addison's disease in a proportion of the study participants and the symptoms at original diagnosis are likely to have been subject to recall bias. The contribution of autoimmunity to the aetiology of Addison's disease may have been underestimated, should patients who were

autoantibody positive at diagnosis, have become autoantibody negative at enrolment in the study.[7 8]

In the absence of a national registry, the prevalence of Addison's disease may have been underestimated. Most patients' lived in close proximity to major teaching hospitals, indicating a likely degree of ascertainment bias. Although the study does show ethnic heterogeneity, the black and Asian participant numbers do not reflect the relative proportions of these groups in the country.[28] Additionally, the study is underpowered to examine HLA differences in these ethnic groups.

This study design also precluded an analysis of lipids and lipoproteins, at diagnosis and in the long-term. It provided a survey of lipid and lipoproteins as a snapshot rather than evaluating the consequences of hypoadrenalism per se and GC replacement therapy, both of which have the potential to influence lipid and lipoprotein metabolism. The TG levels could have been elevated because the samples of South African subjects' lipid profiles were taken in the non-fasted state.[29] In the comparison of the Swedish and South African Addison's patients, lipid analyses were performed in two separate laboratories and exchange of samples was not carried out. Ideally method comparison studies to determine the relative agreement between two different methods should have been carried out.[30]

The healthy South African control subjects were not selected from a central subject pool who meet carefully defined selection criteria, but were recruited from the blood donor clinic. This resulted in inadequate matching, for example there were insufficient black and Asian healthy control subjects and may also have introduced a degree of volunteer bias.[31]

The salivary cortisol study was performed at the participants' homes and was thus unsupervised, allowing for the possibility of erroneous sampling of saliva or failure to adhere to the rigorous requirements of this study. This small study was also

insufficiently powered to determine an association with metabolic derangements.

This relatively large cohort is likely to have been too small and/or too heterogeneous, to find metabolic associations with GCR polymorphisms. Healthy control subjects were limited, precluding perfect matching. As the Addison's patients were receiving empiric GC replacement therapy, selected by their physicians this could have contributed to the lack of association of the GCR polymorphisms and hydrocortisone replacement doses.

8.4 Major implications of this study

It is highly likely that Addison's disease is being under-diagnosed in South Africa given that the prevalence is considerably lower than reported in Western countries.[32-34] and people may be dying despite it being an eminently treatable condition. There should be widespread emphasis on the constellation of specific symptoms to alert physicians to the possibility of Addison's disease and in so doing raise the awareness for this life-threatening disease. The combination of hyperpigmentation, nausea, vomiting and weight loss should suggest Addison'sdisease, as confirmed by three previous studies and corroborated in this study of Addison's disease.[2 35-37] There is a need to develop mindfulness of Addison's disease, which has been referred to as one of the great mimickers of medicine.

A German retrospective study of adrenal insufficiency showed that in 45% of cases a false diagnosis was made, of which psychiatric disorders were thought to be the aetiology in 27%, gastrointestinal disorders in 5% and the remainder were non-classified.[35] The probable explanations for the under-diagnosis include hyperpigmentation which could be overlooked in darkly pigmented races.[38] The large populations of HIV and tuberculosis-infected patients in South Africa may have some of their symptoms erroneously attributed to these conditions,

rather than to primary hypoadrenalism. Under-diagnosis may also be explained by patients having unequal access to health-care, particularly those living in ruralareas. Health delivery is also hindered by the small secondary and tertiary hospital sectors, relative to the need in South Africa.[39 40]

Since Addison's disease occurs even at the highest recorded prevalence at fewer than 5 cases per 10 000 people in the overall population, it may be considered a rare or orphan disease.[41] Most physicians do not see a single patient with a life-threatening rare disease in their entire careers. The European Organisation for Rare Diseases (EURODIS), estimates that there are between 5000 and 8000 distinct rare conditions affecting between 6 and 8% of the population and notes that frequently there are no health policies for these. In addition, there is a paucity of expertise which translates into delayed diagnosis and difficulty in accessing health-care. The benefits of establishing a patient organisation are to create awareness among physicians, to strengthen co-operation with non-governmental organisations and to improve quality of life.[42] Education may be disseminated through websites and local meetings in which patients, their family members, physicians, non-governmental organisation workers and government officials can meet with a view to inform and educate citizens about rare diseases.[43]

Even in the USA, black men suffer worse clinical outcomes compared with other ethnic and gender counterparts. Socio-economic status, masculinity, discrimination on the basis of ethnicity, lack of awareness to seek primary health-care, religious beliefs and influences by peers have been identified as barriers for this sub-group to obtain appropriate and timeous treatment.[44] This phenomenon could have also contributed to the disparity in ethnic representation in the South African Addison's study. Health-care providers and the media could also contribute by raising awareness of either specific illnesses or that all individuals regardless of their ethnicity or gender should be encouraged to seek medical attention. Carnivals may also facilitate dissemination of medical information and

it is the political endorsement of state and national strategies, which is needed to ensure that all ethnic groups and genders do not feel awkward in respect of accessing health-care.[44]

Another possible way to increase recognition of primary hypoadrenalism is to emphasise this condition to medical students. Clinical reasoning is dependent upon two processes: an analytic process which refers to a deductive approach and a non-analytic method or pattern-based recognition, which relies on past clinical experience or illness scripts. Advanced clinicians have inevitably a far more extensive library of illness scripts and rely on pattern recognition.[45] Diagnostic errors have been hypothesised to result from either faulty detection of clinical features or faulty triggering of diagnostic hypotheses. The history and physical examination led to the correct diagnosis in 70%, compared to 35% for radiological techniques in one study, emphasising clinical knowledge and competent reasoning abilities as being pivotal in order to arrive at a correct diagnosis.[46] It was found in 1991 found that 58% of patients attending a rural ambulatory clinic in the USA were subject to a diagnostic error.[45] Checklists, informing colleagues of diagnostic errors, automated decision-making for unusual clusters of signs and symptoms may be helpful in avoiding diagnostic error.[47]

The vital implication of the finding of abnormal lipids, lipoproteins and markers of CVD in primary hypoadrenalism, is that it has elucidated plausible arguments for accelerated CVD mortality in this disease. Addison's disease may now represent an independent CVD risk factor, akin to rheumatoid arthritis, which demands surveillance and intervention.[48] There are several coexistent lipid, lipoprotein and biochemical markers of CVD which corroborate that Addison's disease is at increased risk for CV events. As these markers indicate independent risk, it could attest to the fact that Addison's disease patients may be vulnerable for CVD through multiple concurrent mechanisms. For example, a high proportion of Addison's disease patients had a low HDLC <1.0 mmol/L, which is on its own

strongly predictive for CVD events. By implication a reduction of HDLC by 0.03 mmol/L could accord a 20% increase in CVD events.49 Moreover, approximately 50% of Addison's disease patients exhibited TG >1.7 mmol/L which represents an independent risk factor for CVD. There was a greater proportion of patients, compared to their healthy control subjects, who had predominant small dense LDL, which confers a 3-7 fold increased risk of coronary artery disease.[21]

Physicians should be cognisant of this risk and pay particular attention to screening for CVD risk. This should be incorporated in the usual care of patients, as required in the usual care of a diabetic patient.[50] Lifestyle modification, exercise and dietary recommendations should be the norm and a low threshold for introduction of lipid-modifying therapy and a preparedness to confirm that the NCEP ATP III targets are being achieved.[51] The patients who have both diabetes and Addison's disease will require meticulous surveillance and intervention to ensure that their additional CVD risk factors, such as adequate glycaemic and blood pressure control, are addressed.

This study should serve as a reminder to clinicians that replacement doses of hydrocortisone may induce supra-physiological concentrations of cortisol, potentially placing patients at risk for the cluster of GC related side-effects. A systematic evaluation for possible GC excess should include evaluation of emotional lability, weight gain, insomnia, evolution of diabetes mellitus, hypertension, peptic ulcer disease, impaired wound healing, increased susceptibility to infections, atherosclerosis, fatty liver, osteoporosis, glaucoma and mood disturbance.[52]

As the ER22/23EK polymorphism was associated with an elevated BMI, this could contribute to the increased CVD risk. Analysis of the Framingham data identified that the relative risk for CVD for the overweight category was 1.20 and the relative risk for the obese males and females was 1.46 and 1.64 respectively, after adjusting for age,[53] emphasising the importance of increased weight as a

CV risk factor. Routine assessment of body mass and anthropometry at patient follow-up visits should be recommended in Addison's disease patients.

8.5 Recommendations for future study

As Addison's disease is rare, collaboration with established research groups may provide sufficient numbers of patients and thus greater statistical power to address research questions in relation to CVD risk, salivary cortisol exposure and the influence of GCR polymorphisms, raised by this study. Several international Addison's disease registries have already contributed substantially to the body of knowledge relating to this disease. For example Euradrenal is the European patient registry, representing multiple European nations' cohorts of autoimmune Addison's disease and incorporating several national networks. From 2009 to 2010, at least 17 publications were identified from this registry, covering immunology, autoimmunity, metabolic and CV profiles, clinical features of primary hypoadrenalism and complications related its replacement.[54] Registries such as these would provide ideal platforms with which to collaborate.

8.5.1 Suggestions for further evaluation of CVD risk

Further evaluation of CVD risk in Addison's patients should be undertaken using the national registry and in collaboration with international research groups. Utilising a national database, dietary evaluation could elucidate dietary influences on lipids and lipoprotein and may explain the vast differences between the Swedish and South African cohorts. Dietary patterns have been used to examine the relationship of health outcomes and the total usual food intake in individuals and groups. Multiple diet-record days, 24-hour recalls or the Healthy Eating Index score may be used to assess consumption.[55] The latter score allocates points for saturated fat intake, and intake of fruit, vegetables, grains, milk and dairy products. The Mediterranean diet score evaluates the intake of non-refined cereals, fruits, vegetables, potatoes, legumes, olive oil, fish, red meat, poultry, full

fat dairy products and alcohol. Statistical analyses examine actual intake patterns and have been shown to offer good reproducibility in diverse populations.[55] Such analysis could be useful in understanding if lifestyle contributes to worse lipid and lipoprotein profiles in South Africa compared to Sweden. On the other hand, it should be appreciated that dietary analyses require large numbers of participants for the association of CV risk with eating patterns to be demonstrated. The association in several studies was attenuated after adjusting for several factors including smoking, physical activity, education level, BMI and alcohol consumption.[56]

Although collaboration with international research groups could improve follow-up and documentation of CVD events and it may permit the derivation of a conversion factor for the Framingham risk assessment of Addison's disease patients, a 12 year follow-up possibly is warranted with several thousand Addison's patients. Identification that hs-CRP is elevated in Addison's disease in this cohort should be a catalyst to study whether this translates into imminent risk for CVD events or whether it is a signal for emerging serious infection. In the setting of community acquired pneumonia for example, hs-CRP has an important role in distinguishing bacterial from non-bacterial and non-infectious causes of respiratory symptoms, supporting its role as an early warning system for serious infection.[57] Particularly, when examining CVD events large numbers of participants in association with Addison's disease are required to generate meaningful data, but designers of these kinds of studies should be mindful of the local background event rates and adjustaccordingly.

There are several further areas of study relating to CVD which warrant evaluation requiring large-scale cooperation with international research groups. In order to gain insights as to whether the lipid abnormalities may be associated with Addison's disease and/or its treatment, it would be crucial to evaluate lipids and lipoproteins at diagnosis prior to initiating GCs and then by later follow-up

studies once established on maintenance GC therapy. Ridker et al, in 2003 showed that the relative risk for first-ever myocardial infarction or stroke as well as the onset of symptomatic peripheral arterial disease relates to baseline hs-CRP.[58] Epidemiological studies have confirmed a strong relationship between hs-CRP and carotid intimal medial thickness, warranting confirmation whether this relationship also occurs in Addison's disease, to account for this increased CV mortality.[59] The implication of NEFA concentrations should also be examined in future studies to determine if lower concentrations have a protective effect against future CV events.

8.5.2 Suggestions for further evaluation of salivary cortisol

Larger international studies are required to corroborate the discrepant cortisol exposure in Addison's disease patients on hydrocortisone and healthy control subjects' endogenous cortisol concentrations noted in this study, and whether this excessive exposure predisposes to the evolution of complications. Further studies are warranted to assess whether a dose reduction of hydrocortisone could minimise the development of side-effects. As the peak salivary cortisol correlates well with the AUC, this relationship should also be explored to determine if a correlation exists with metabolic consequences.

While Addison's disease patients have subjective impaired health quality attributed to the prolonged nadir of sub-physiological concentrations of serum cortisol,[60] depression has also been linked to increased salivary cortisol exposure.

In the context of Addison's disease patients exhibiting higher salivary cortisol concentrations it would be important to establish whether there is a correlation between salivary cortisol concentrations and depression scores.[61] Psychological well being scores and correlation with salivary cortisol AUC could clarify, whether supra-physiological doses of hydrocortisone may be contributing to depression, rather than the prolonged nadir as previously reported.60 Such a study could be

performed using both an available national registry of Addison's disease and in collaboration with others.

As there is progress in the development of newer modified prolonged release preparations for replacing GCs in hypoadrenal individuals as reported by Johannsson et al,[62] salivary cortisol could be an additional modality in which to evaluate whether the exposure to cortisol is more physiological.

8.5.3 Suggestions for further genetic analyses

International larger studies could confirm the phenotypic findings in relation to the GCR polymorphisms. Determination as to whether the GCR polymorphisms confer subtle derangements in insulin sensitivity parameters could be of interest as insulin resistance may potentiate CVD risk.[63] Enzyme kinetics of 11 beta-hydroxysteroid-dehydrogenase-I may influence the clinical phenotype. Two polymorphisms rs846910 and rs13306421 have an in vitro effect of raising this enzyme activity and evaluation should be undertaken as to whether these polymorphisms associate with metabolic abnormalities in Addison's disease. Potentially they can enhance the GC side-effect profile of replacement doses, by reducing metabolism to cortisone.[64] Larger cohort studies will make inferences of genetic conclusions far more credible.

8.5.4 Novel forms of therapy

While there is ongoing research aimed at improving replacement therapy for patients with Addison's disease,[62] these novel forms of therapy may need to be evaluated with GCR or 11 beta-hydroxysteroid-dehydrogenase-I polymorphisms, with the view of developing individualised, rather than empiric therapy.

8.5.5 Broader consequences of this study

The methodology employed in this study to identify patients with a rare disease could serve as a prototype for identifying rare diseases in South Africa, until

databases are actualised. This study has underscored that there is a failure to institute global recommendations, for example, initiating lipid-modifying therapy in diabetics and it has documented that even when this therapy was administered, the minority achieved targets. Studies have shown that although patients were prescribed lipid-lowering agents, many patients failed to achieve adequate goals due to poor adherence, affordability of therapy and factors relating to health-care provision including either not following or understanding the guidelines, inadequate follow-up with laboratory testing, and insufficient dose-titration. Despite these barriers, a recent study revealed that close follow-up and point of care testing of lipids and lipoprotein fractions resulted in 68% of participants maintaining target lipid levels for three years compared to only 30% in other national surveys.[65]

The South African Addison's study has also highlighted the significant deficiencies in current local available normative lipid data. This should be prioritised and should include populations of HIV-infected patients. These patients are prone to decreases in HDLC and LDLC and those who are treated with highly active anti-retroviral therapy have a propensity to exhibit elevations in TC, LDLC and HDLC with first-generation nonnucleoside reverse transcriptase inhibitors, while the second generation nonnucleoside reverse transcriptase inhibitors have less effect than the first generation. The nucleoside reverse transcriptase inhibitors impact on both TG and TC and protease inhibitors have led to enhanced VLDL secretion.[66] Therefore HIV positive patients and those who are on antiretroviral therapy should be screened for lipid and lipoprotein abnormalities, as they represent a large population which could influence national averages.

While hypopituitarism has enjoyed extensive recognition as a condition associated with CVD risk, there have been very few reports of the relationship between lipid abnormalities and CVD in Addison's disease and CAH.[67-69] Appreciation for repeat lipid and lipoprotein evaluation in Addison's disease and a low threshold to intervene is warranted. As salivary cortisol is markedly elevated in Addison's

disease patients, compared with healthy control subjects' endogenous cortisol concentrations, this study should be extended to evaluate CAH, hypopituitarism and asthma sufferers. Similarly, GCR polymorphisms should also be evaluated and correlated with hydrocortisone dose, in the setting of hypopituitarism and CAH to determine if they have an impact on metabolic parameters or doses of hydrocortisone.

8.6 Final conclusions

An extensive analysis of Addison's disease in South Africa has been undertaken and has yielded novel data. It has highlighted the need for improved education of medical students and practising doctors so that the diagnosis of Addison's disease is not missed. There should be emphasis on the constellation of symptoms suggestive of primary hypoadrenalism to remind clinicians of this disorder. Moreover it should alert the clinician to consider Addison's disease as a condition, requiring surveillance and intervention to limit CV risk. It highlighted that the cortisol exposure in hydrocortisone replaced patients is many-fold higher than healthy control subjects, necessitating both further studies and appreciation for the potential harm. Further studies, incorporating cohorts from different parts of the world should be undertaken to corroborate and extend the findings of this body of work.

8.7 References

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